STUDIES ON INTERFERON PRODUCTION

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1940

A DISSERTATION PRESENTED TO THE GRADUATE COUNCIL OF
THE UNIVERSITY OF FLORIDA
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE
DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA
1969

ACKNOWLEDGMENTS

I acknowledge my deepest appreciation to Dr. G. E. Gifford for his encouragement, supervision and guidance throughout the period of this investigation and during the preparation of the manuscript. I also thank the members of my advisory committee, Dr. H. E. Kaufman, Dr. L. W. Clem and Dr. P. Byvoet for their suggestions and encouragement. I am grateful to Dr. P. A. Small for critically reviewing the manuscript. I would like to thank Dr. I. Rosen who helped me in many ways during my studies.

I would also like to thank Mrs. P. Jones for the generous supply of cell cultures and other materials. I am also grateful to Mr. M. Fruitstone, Mrs. B. Asch, Mrs. J. Curry and fellow graduate students for their suggestions, aid and criticisms during this investigation.

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KEY TO ABBREVIATIONS

The following abbreviations were used in the text.

BSS	Balanced salt solution
CAM	Chorioallantoic membrane
CE	Chick embryo
CS	Calf serum
DNA	Deoxyribonucleic acid
FPA	Fluorophenyalanine
MEM	Minimum essential medium
MM	Maintenance medium
MMM	Modified maintenance medium
m-RNA	Messenger ribonucleic acid
NDV	Newcastle disease virus
pi	Post infection
PDD_{50}	50 per cent plaque depressing dose
pfu	Plaque forming unit
rA/rU	Riboadenylic acid/ribouridylic acid
rI/rC	Riboinosinic acid/ribocytidylic acid
RNA	Ribonucleic acid
RNAse	Ribonuclease
RSB	Rabbit reticulocyte buffer

SDS	Sodium dodecyl sulphate
S	Sedimentation coefficient
TIP	Translational inhibitory protein
VSV	Vesicular stomatitis virus
W/V	Weight per volume

INTRODUCTION

One of the intriguing aspects of viral induced interferon production is the role of multiplicity of infection. It has been known that high multiplicities of certain viruses inhibit interferon production while higher yields of interferon are obtained with lower multiplicities of infection. (De Maeyer and De Somer, 1962; Gifford, 1963; Aurelian and Roizman, 1965). Similar results were reported by Toy and Gifford (1967a) who observed that maximal amount of interferon was obtained in Semliki Forest virus (SFV) infected chick embryo (CE) cells at a multiplicity of 0.2 pfu/cell while multiplicity of 10 pfu/cell resulted in marked reduction of interferon yield. An attempt was made to explain this phenomenon by studying the effect of these two multiplicities of infection on host cell macromolecular synthesis in the infected cells. The study of RNA synthesis was suitable since cellular RNA could be differentiated from viral RNA by the use of actinomycin D, an antibiotic which is known to inhibit cellular RNA synthesis but has no effect on Semliki Forest virus replication or its RNA synthesis. The other factors considered in this study were: (1) effect of progeny virus on interferon production, and (2) production of interferon by heat inactivated virus.

The physico-chemical nature of the interferon inducers has, so

far, proved to be elusive. Recently, several investigators (Field et al., 1967; Vilcek et al., 1968; Dianzani et al., 1968) have reported that interferon production can be induced by complexes of polyriboinosinic acid and polyribocytidylic acid (rI/rC) and other synthetic, double stranded RNA preparations, in vivo as well as in vitro. However, the role of single stranded RNA in interferon induction has been a subject of controversy. Field et al. (1967) reported that single stranded RNA did not induce interferon production. These results have been confirmed by Vilcek et al. (1968), Tytell et al. (1967) and Hilleman (1969). However, Baron et al. (1968) and De Clercq and Merigan (1969a) found that certain single stranded polyribonucleotide preparations such as polyriboinosinic acid and polyribogunylic acid were able to induce small amounts of interferon. Since heat inactivated SFV, though unable to replicate, induces interferon production, the question that naturally presents itself is: does the heat inactivated noninfectious SFV synthesize a double stranded, replicative form when it induces interferon production in CE cells or is the single stranded viral RNA in itself the inducing agent? Part of the dissertation deals with an attempt to answer this question.

The antiviral action of interferon in cells is due to the de novo synthesis of another protein which apparently confers on ribosomes the capability to distinguish between the cellular and viral messenger RNA. These ribosomes are capable of forming polysomes with cellular messenger RNA but bind poorly to viral messenger RNA and thus

the production of viral RNA directed proteins is inhibited (Marcus and Salb, 1966; Carter and Levy, 1967). However, it is not known whether this interferon induced protein acts alone or in conjunction with interferon. If interferon does play a direct role in the maintenance of a virus resistant state in the cells, it is reasonable to assume that it would also combine with ribosomes for the expression of antiviral activity. To investigate this possibility, chick embryo and mouse liver ribosomes and their subunits were exposed to various interferon preparations and were examined for their capability to combine with interferon.

REVIEW OF LITERATURE

Interferon is a term given to a group of antiviral proteins that can be induced in vivo and in vitro by a wide variety of agents. It was first described by Isaacs and Lindenmann (1957) as a protective factor against viral replication in chorio-allantoic membranes induced by heat inactivated influenza virus. Since then, interferon has been induced by every major group of viruses and by a variety of nonviral inducers of interferon-like substances. Thus, it has become evident that production of interferon by cells is a general nonspecific response to various stimuli. Interferons, in general, can be divided into two broad classes. (1) Interferons whose synthesis is dependent upon RNA and protein synthesis in the induced cells. Interferons produced in response to viruses form a major group in this class. (2) Interferons whose appearance after induction is not dependent upon RNA and protein synthesis and seem to be released from certain cells (preformed interferon). The other differences in the two classes of interferons are summarized in Table 1. However, some inducers may invoke both types of response and characterization of such preparations of interferon becomes difficult.

The inhibition of viral induced interferon production by actinomycin D was first reported by Heller (1963) and Wagner (1963). This finding

TABLE 1

COMPARISON OF VIRAL AND NON-VIRAL INDUCED INTERFERONS

Characteristics	Viral	Non-viral	Reference
Kinetics of production (serum)	Late peak	Early peak	Youngner, J., Hallum, J., and Stinebring, W. (1966)
Molecular weight (serum)	often > 50,000	often < 50,000	Ibid.
Sensitivity of Production:			
Protein synthesis inhibitors	sensitive	not sensitive	Ho, M., and Breinig, M. K. (1965)
Actinomycin D	sensitive	not sensitive	Finkelstein, M. S., Bausek, G. H., and Merigan, T. C. (1968)
Presence of spleen during induction	not essential	essential	De Somer, P., and Billiau, A. (1966)
Effect of BCG sensitization on production	no influence	increased production	Youngner, J., Hallum, J., and Stinebring, W. (1966)
Elevated temperature	increased production	no influence	Isaacs, A. (1961)--viral, De Somer, P., and Billiau, A. (1966)--non-viral

was interpreted as evidence for the coding of interferon by the cellular genome rather than by the inducer virus. A similar effect of other DNA dependent RNA synthesis inhibitors like 4, 5, 6-trichloro-1- D-ribofuranosyl benzimidazole and 8-azaguanine on interferon synthesis has been reported by Walters, Burke and Skehel (1967). The other lines of evidence which support this hypothesis are provided by the observations that (1) interferons are species specific, and (2) DNA as well as RNA viruses induced apparently identical interferons in chick cells (Lampson et al., 1965). Thus, it seems that in interferon induction by viruses, a stimulus leads to cell directed RNA synthesis which then controls the synthesis of the new protein, presumably different from the other proteins of the cell.

The concept of viral induced interferon formation as a newly synthesized protein is supported by the work of Buchan and Burke (1965, 1966) who reported that 25 μ g/ml of p-flurophenylalanine, in phenylalanine-free medium, completely inhibited subsequent interferon formation in chick embryos exposed to ultraviolet-inactivated influenza virus. This substance acts as an antagonist of phenylalanine and is incorporated into proteins. Thus, the proteins might have a reduced or no biological activity depending upon the importance of the phenylalanine residue for biological activity (Richmond, 1960, 1963). Similarly, puromycin at low concentrations has also been found to completely inhibit interferon production in various cell-virus systems. It was reported by Wagner and Huang (1965) that once interferon

production has started in the Krebs-2 carcinoma cells-Newcastle disease virus (NDV) system, the addition of puromycin resulted in the immediate cessation of interferon production. Thus, the rate at which interferon appears in cell culture fluids reflects the actual rate of synthesis and there seems to be no build-up of the product in the cells. Similar results have been obtained by Buchan and Burke (1965, 1966) employing chick embryos infected with ultraviolet-inactivated virus.

This overall similarity of interferon production with Jacob and Monod's operon model led Burke (1966) to propose a hypothetical scheme in which virus invasion is presumably followed by uncoating and release of an interferon inducer which induces the production of interferon specific messenger-RNA. This m-RNA then directs the synthesis of interferon. However, there is no well established case for the operon model's counterpart in the mammalian system. In the interferon system, the exact nature of the inducer, the mechanism of interaction between inducer and host genome, and the nature of the repressor molecule, if present, are largely unknown. Attempts to obtain direct evidence for interferon specific messenger RNA have not been successful (Burke and Low, 1965; Wagner and Huang, 1966). However, Wagner and Huang (1965, 1966) reported that in the Krebs-2 carcinoma cell-NDV system, practically all the interferon specific messenger is synthesized within 6 hours after infection and is relatively stable for at least 10 hours.

The discovery of substances other than viruses that can elicit interferon production was first reported by Isaacs, Cox and Rotem (1963). They found that chick, mouse and rabbit ribosomal RNA did not induce interferon in homologous cells unless the RNA was first made "foreign" by treatment with nitrous acid. Ribonucleic acid from E. coli, turnip yellow mosaic virus and rat liver DNA though "foreign" were not effective inducers of interferon in chick cells. Rotem, Cox and Isaacs (1963) also showed that chick liver RNA inhibited the growth of vaccinia virus in mouse cell cultures and mouse liver RNA inhibited vaccinia virus in chick cell cultures.

Youngner and Stinebring (1964) reported the production of low amount of interferon in chickens infected with Brucella abortus. Stinebring and Youngner (1964) also found that intravenous injections of endotoxin, Salmonella typhimurium, or Serratia marcescens caused a rapid appearance of interferon in the blood stream of mice. Ho (1964) also reported the production of interferon in rabbits injected with endotoxin. Youngner and Stinebring (1965) found that endotoxin or Brucella abortus induced interferon formation was unaffected by concentrations of actinomycin and cyclohexamide which inhibited more than 97% of RNA and protein synthesis, respectively, in mouse liver. They also observed that pretreatment of animals with puromycin or cyclohexamide enhanced and prolonged the interferon response. Gifford (1965) found that yeast RNA, hydrolysate of RNA, and mononucleotides inhibited vaccinia and chikungunya virus in cell cultures but interferon

was not present in high enough titers to account for all the viral inhibitory effects. Field et al. (1967) reported the formation of interferon in rabbits and their spleen cell cultures after induction by synthetic polyribonucleotides. Only double stranded polyribonucleotides, such as polyinosinic acid/polycytidylic acid (rI/rC), were found to be effective inducers while double stranded DNA polymers or single stranded RNA polymers were unable to elicit an interferon response. Similar results have also been obtained by Vilcek et al. (1968), Falcoff and Bercoff (1968), Tytell et al. (1968), and Field et al. (1968).

However, it has also been claimed that single stranded polyribocytidylic acid as well as polyriboinosinic acid can induce interferon formation (Baron et al., 1968; Levy et al., 1968; Finkelstein, Bausek and Merigan, 1968). It was then suggested that the inducing ability of single stranded polyribonucleotides may be due to the contamination of these preparations with double stranded polymers (Field et al., 1968; Tytell et al., 1968). But De Clercq and Merigan (1969a) reported that the single stranded homopolymers, polyriboguanylic acid, polyriboinosinic acid and polyriboxanthylic acid, at neutral pH and polyriboadenylic acid and polyribocytidylic acid at acid pH can induce interferon production. The preparations were apparently free of double stranded polymers and the inducibility characteristic was related to the stability of the secondary structure of homopolymers as indicated by their high temperature of melting (Tm) values. However, triple stranded complexes of polyribonucleotides, having higher Tm values,

were much less active than the double stranded polymers. It was postulated that differences in the inductive capability of various polyribonucleotides may be due to the differences in their affinity for the site of initial interferon formation inside the cells, even though cells seem to have similar permeabilities to various effective and ineffective RNA polymers (Field, Tytell and Hilleman, 1969) and such polymers are equally suceptible to nuclease degradation inside the cells (Colby and Chamberlin, 1969). De Clercq, Eckstein and Merigan (1969) recently reported that substitution of the phosphate group by a thiophosphate group in an alternating copolymer, rA/rU, resulted in a 2-20 fold increase in its ability to induce interferon production. This increase in inducibility was accompanied by a 10-100 fold increase in resistance to pancreatic ribonuclease. It has been suggested (De Clercq and Merigan, 1969b) that the common structural requirement for polyribonucleotides and other synthetic polyanion interferon inducers include (a) high molecular weight, (b) a stable, primary and long, carbon to carbon backbone, and (c) a regular and dense sequence of negative charges on the backbone. It has been found by several investigators that polybasic substances such as protamine sulphate, neomycin sulphate and DEAE dextran significantly increase the yields of interferon in vitro as well as in in vitro (Vilcek et al., 1968; Falcoff and Bercoff, 1968) by polyribonucleotides. The mechanism of enhancement is not well understood although it has been postulated that enhancement is due to the increased penetrability of the polyribonucleotides into the cells.

The effect of actinomycin D on polyribonucleotide induced interferon formation has been a subject of controversy. Vilcek et al. (1968) observed that 2 μ g/ml of actinomycin D inhibited the production of interferon in rabbit kidney cells induced by poly rI/rC. Similar results were obtained by Falcoff and Bercoff (1968) in human leukocytes and in amniotic membranes. However, Finkelstein, Bausek and Merigan (1968) observed that the concentration of actinomycin D which suppressed NDV-induced interferon production had no effect on the interferon formation induced by poly rI/rC, pyran, and endotoxin in human skin fibroblast and mouse peritoneal macrophage cultures. It was also found that actinomycin suppressed polyribonucleotide-induced interferon formation only at concentrations which were cytotoxic. Vilcek, Rossman and Varacalli (1969) observed that actinomycin D, if applied two hours or later after the induction with poly rI/rC, does not affect the yields of interferon in rabbit kidney cell cultures. The formation of polyribonucleotides-induced interferon is not inhibited by protein synthesis inhibitors like p-flurophenylalanine and puromycin in vitro (Finkelstein, Bausek and Merigan, 1968) or in vivo (Youngner and Hallum, 1968) while viral-induced interferon synthesis was suppressed under similar conditions.

Differences between the two classes of interferons (summarized in Table 1) raises the question of whether or not the mechanism of induction in both classes (preformed versus synthesized) is similar. The question becomes more pertinent since the two responses also occur

in morphologically uniform population of cells like human skin fibroblast indicating that the same cell is capable of responding in two different ways. It has been postulated that there may be two different processes of interferon induction, i. e., de novo synthesis as well as release of already formed interferon (Ho and Kono, 1965; Ho, Postic and Ke, 1968; Youngner, 1968; Finkelstein, Bausek and Merigan, 1968). It has also been suggested by Finkelstein, Bausek and Merigan (1968) that both responses may involve de novo synthesis but that one requires an additional step, e. g., virus uncoating which is sensitive to metabolic inhibitors; or, that both responses may require de novo interferon synthesis and both have similar biological activity but possess different sensitivities to metabolic inhibitors and are released or formed at different rates. Youngner (1968) suggested that the two responses may result in release of preformed interferon but the viral-induced type may require an interferon activating enzyme whose synthesis is suppressed by metabolic inhibitors. However, this possibility is excluded by the work of Paucker (1969) who reported, on the basis of radiolabelled pulse experiments, that interferon produced in L cells by UV-unactivated NDV is essentially a product of de novo synthesis. A definite explanation for the mechanism of the two types of responses may be possible once radiolabelled amino acid incorporation studies on polyribonucleotide induced-interferon become available.

Production of Interferon

Interferon is a product of the interaction of cell and inducer. Both

elements of interaction seem to be equally important determinants of whether interferon is produced and how much. Production would also be dependent upon the type of cellular environment in which this interaction takes place.

Cellular Aspects of Interferon Production

Genetic control

The direct evidence for the genetic control of interferon production by cells comes from the observation of Desmyter, Melnick and Rawls (1968) that Vero cells, a line of African green monkey kidney cells, was unable to produce interferon when infected with NDV, Sendai, Sindbis or Rubella virus. This apparently qualitative defectiveness in interferon production has also been noted when Vero cells were exposed to poly rI/rC with or without protamine sulphate or neomycin sulphate (Schaffer and Lockart, 1969). The cell line, though incapable of producing interferon, is sensitive to the action of exogenous interferon. There is no information in regard to the size of the locus or the number of genes involved in this defectiveness. Cogniaux-Le Clerc, Levy and Wagner (1966) reported that the loss of ability to produce interferon as a function of increasing UV dose showed first order kinetics and it was suggested that a single site on cellular DNA controls the formation of the interferon specific messenger RNA. Similar results were obtained by Burke and Morrison (1966).

Types of cellular response

Endotoxin, when administered into intact animals, elicits significant

amounts of interferon but is unable to induce interferon in vitro except in leukocytic cell cultures (Smith and Wagner, 1967). Lackovic et al. (1967) reported failure of L cells to produce interferon when treated with mannan, a yeast polysaccharide, although a good response was obtained with mouse peritoneal macrophage cultures. Similarly, Finkelstein, Bausek and Merigan (1968) observed that pyran, a polycarboxylate polymer, and poly rI/rC induced interferon in mouse peritoneal macrophages but not in L cells. Thus, it appears that there are several types of cellular responses to inducers, with aneuploid cell lines being most restrictive. The type of cellular response, at least in part, seems to depend upon the penetrability of the inducer into the cell since L cells have been reported to produce significant amount of interferon with poly rI/rC after prior treatment with diethylaminoethyl (DEAE) dextran (Dianzani et al., 1968). Other factors which may contribute to variation in types of cellular responses have not been elucidated.

Host macromolecular synthesis

The evidence available indicates that synthesis of interferon is a latent cellular function, induced by viral infection or certain nonviral agents. Thus, the role of cellular macromolecular synthesis in the production of interferon becomes obvious. Several investigators have reported that virulent viruses induce very low yields of interferon. Ruiz-Gomez and Isaacs (1963) reported that NDV, a cytopathic virus in CE cells, grew in high titers but produced very little interferon.

However, when mouse embryo fibroblast or human thyroid cell cultures were infected with the same virus, no cytopathology was observed and high yields of interferon were obtained. Similar results were obtained with vesicular stomatitis virus (VSV) (Wagner et al., 1963), foot and mouth disease virus (Sellers, 1963, 1964), polyoma virus (Friedman and Rabson, 1964) and various arboviruses (Ruiz-Gomez and Isaacs, 1963). Virulent myxovirus has been reported to inhibit interferon synthesis induced by UV-inactivated (Lindenmann, 1960) or avirulent myxoviruses (Hermodsson, 1963). However, there is not always a correlation between the virulence of virus and interferon production: e.g., some strains of influenza virus (Inglot, Kochman and Mastalerz, 1965; Link et al., 1965a), Sindbis virus (Vilcek, 1964), vaccinia virus (Link et al., 1965b) and NDV (Baron, 1964) do not follow this pattern.

The mechanism by which certain viruses can inhibit interferon production was investigated by Wagner and Huang (1966). They induced interferon production in suspension cultures of Krebs-2 carcinoma cells by employing an avirulent strain of NDV. Interferon was first detected 3 to 4 hours after the infection and production increased almost linearly, reaching a peak at 20 hours. However, interferon synthesis was terminated when cells were superinfected with VSV (50 pfu/cell) within 4 hours after infection with NDV. Vesicular stomatitis virus does not induce interferon in this cell system. Krebs-2 carcinoma cells infected with VSV exhibited a rapid and almost immediate decline in the rate of cellular RNA synthesis but NDV, at the

multiplicity employed, had no appreciable effect for at least 3 hours after infection. Therefore, it was postulated that VSV prevented interferon synthesis by inhibiting cellular RNA synthesis. Similar results were reported by Aurelian and Roizman (1965) employing a strain of herpes virus which caused an abortive infection in dog kidney cells. Infection at a multiplicity of 100 pfu/cell led to the formation of viral DNA and antigen without any production of interferon, whereas infection at lower multiplicity (12 pfu/cell) resulted in the production of interferon, but not virus. In the former case, virus infection caused a rapid decline in cellular RNA synthesis and, hence, the cessation of interferon production. At the lower multiplicity, virus induced inhibition of RNA synthesis did not occur until 5 to 6 hours after infection and interferon production was thus permitted.

Bolognesi and Wilson (1966) have reported a rapid and profound decline in the rate of cellular protein synthesis in NDV infected CE cells. Most probably, marked inhibition of protein synthesis is the reason for the lack of interferon production in this system. The inhibition of protein synthesis can be ascribed to either reduction in cellular RNA synthesis or inhibition of messenger RNA translation in NDV infected CE cells. However, the relationship between interferon production and the rate of cellular protein synthesis is complex; Friedman (1966a) observed that interferon yields were reduced in SFV infected CE cells under conditions when overall cell protein synthesis was augmented and postulated that increased protein synthesis resulted

in the accumulation of an interferon repressor in the cells thereby decreasing interferon yields.

Factors Influencing Interferon Production

All the conditions which favor the optimal production of interferon are not yet known. However, there are some factors which have been reported to influence interferon synthesis and are considered in this section.

Priming

The priming effect is obtained when cells treated either with inactivated virus or with interferon respond by an enhanced production of interferon on subsequent induction. Burke and Isaacs (1958) observed that influenza virus could induce interferon formation in chick embryo chorio-allantoic membrane only if the tissue had first been primed with heat-inactivated virus. Similar results have been obtained with Eastern equine encephalitis virus (Mahdy and Ho, 1964) and Sindbis virus (Ho and Breinig, 1962). Isaacs and Burke (1958) also described the potentiating effect of interferon on subsequent interferon formation. However, Vilcek and Rada (1962) and Paucker and Cantell (1963) reported that pretreatment of cells with interferon inhibited the subsequent yields of interferon when challenged with a viral inducer. Later it was found (Lockart, 1963; Taylor, 1964; Friedman, 1966b) that pretreatment of cells with interferon may indeed have both effects depending upon the amount used and multiplicities of virus subsequently employed to elicit interferon production. Levy, Buckler and Baron (1966) and

Friedman (1966b) showed that "priming" not only increased the yields but interferon was synthesized more rapidly in the primed cells. Friedman (1966b) has also shown that enhancement of interferon production is dependent upon active protein synthesis in the cells during the priming period. However, synthesis of interferon specific messenger RNA was not detected during the period of priming. It was also reported that interferon-cell interaction for priming could occur at 4°C but would be expressed only if cells were further incubated at 37°C for several hours before challenging with virus.

Temperature

Interferon production can only occur within a certain range of temperature as would be expected for any cellular metabolic process. Its synthesis was inhibited when cells were incubated at 4°C (Isaacs, 1963). Ruiz-Gomez and Isaacs (1963a, b) observed that the optimum temperature for interferon production was generally higher than that for virus replication. For example, more interferon was induced by chikungunya virus at 42°C, an unfavorable temperature for virus replication, than at 39°C or 35°C. Burke, Skehel and Low (1967) found that SFV induced optimal yields of interferon at 42°C when no virus replication was detected. However, the cells have to be pre-incubated with virus at 37°C for some time to presumably allow its adsorption and uncoating before shifting the temperature to 42°C. Ruiz-Gomez and Isaacs (1963) also suggested that this increased interferon production at higher temperature may constitute a defense

mechanism which prevents the pathogenic effect of viruses during febrile conditions. The results obtained by Ruiz-Gomez and Sosa-Martinez (1965) tend to support this hypothesis. Mice inoculated with Coxsackie B virus were kept at 4°C, 11°C and 25°C. Those kept at 4°C developed viremia with high titers in heart and liver and died 3 to 4 days after infection. Their livers had very little interferon except on the first day after infection. However, the mice kept at 25°C survived, virus did not replicate in any tissue, and livers contained high titers of interferon.

The other aspect of this relationship was reported by Siegert, Shu and Kholhage (1967) who found that production of interferon in rabbits infected with myxovirus was accompanied by fever. Similarly, Merigan (1968) reported that interferon inducing doses of pyran also caused fever in man. However, this correlation was not observed with measles vaccine which induced interferon production without concomittant induction of fever in human beings. Conversely, bacterial endotoxin or etiocholanalone induced fever without the production of interferon. However, endotoxin which is capable of inducing interferon production in mice (Stinebring and Youngner, 1964) as well as in rabbits (Ho, 1964) is also known to produce fever in rabbits.

Multiplicity of infection

In several instances of cell-virus interaction, the multiplicity of infection seems to play a significant role in determining the amount of interferon synthesized by the induced cells. De Maeyer and De Somer

(1962) reported that rat tumor cells infected with Sindbis virus produced maximal amounts of interferon when a multiplicity of 0.1 pfu/cell was employed, and much lower yields were obtained at a multiplicity of 10 pfu/cell. Gifford (1963) observed that chikungunya virus best induced interferon production when the input multiplicity of infection was 0.1 pfu/cell while higher multiplicity reduced the yields of interferon. The observations of Aurelian and Roizman (1965) in the dog kidney cell-herpes virus system and Toy and Gifford (1967a) in the SFV-CE system tend to support the concept that higher multiplicity of infection often inhibits interferon production whereas more interferon is produced with lower multiplicities.

Nature of Inducing Agent

The physico-chemical nature of the agents which induce interferon has not been well elucidated. Isaacs (1961) suggested that the induction of interferon by viruses might be due to their "foreign" nucleic acid. This concept was in agreement with the observations that viruses, composed of nucleic acid and protein only, could induce interferon production, but incomplete viruses containing less nucleic acid failed to induce.

Low yields of interferon have been obtained in various cell systems by Rotem, Cox and Isaacs (1963) with heterologous RNA, and by Isaacs, Cox and Rotem (1963) using nitrous acid treated homologous ribosomal RNA. The few known properties of nucleic acid-induced viral inhibitors suggested identity with viral-induced interferon, but the physico-chemical characterization of these preparations has not been carried

out in detail. Moreover, several investigators were unable to confirm these findings. However, the work of Skehel and Burke (1968a) supports the hypothesis that complete viral nucleic acid is essential for the induction of interferon production. These authors studied the effect of hydroxylamine on SFV. Hydroxylamine has been extensively employed to inactivate the infectivity of viruses by reacting with viral nucleic acid; it has no effect on viral antigenicity (Schafer and Rott, 1962). The inactivation of SFV by 0.2 M hydroxylamine at 25°C followed a pattern of first order kinetics, and the interferon-inducing capacity decreased at the same rate as that of infectivity. There was no effect on hemagglutination titers, indicating that the protein coat of the virus was unaffected by the treatment. These results confirmed the significance of viral nucleic acid in the process of induction but did not rule out the possibility that viral protein(s) may also be essential for interferon induction.

Burke, Skehel and Low (1967) studied the early stages of interferon induction. The system employed was infection of chick cells with SFV for one hour at 36°C followed by shifting the temperature to 42°C. Under these conditions interferon is produced but the virus does not replicate. It was found that during the incubation period at 36°C some ribonuclease resistant RNA was synthesized in the infected cells (Skehel and Burke, 1968b), and it was suggested that formation of a double stranded, replicative form of viral RNA may be the first step in induction of interferon formation. Once again, the role of

viral protein(s), if any, could not be ascertained.

Double stranded polyribonucleotides have been reported to induce interferon production in vivo as well as in vitro but the role of single stranded polyribonucleotides in interferon induction has not been clearly established (discussed on pages 9,10, and11). However, it has been reported that nucleic acid preparations release preformed interferon (Finkelstein, Bausek, Merigan, 1968; Youngner, 1968), unlike the viral-induced interferon which is synthesized de novo. Therefore, it is a distinct possibility that induction for the two types of responses may not be achieved through the same process.

Lockart et al. (1968) employed temperature sensitive mutants of Sindbis virus in CE cells for interferon induction. The authors concluded that input viral RNA and the replication of viral RNA are not sufficient for interferon induction but the induction event requires some viral protein(s) or the process for which these proteins are necessary. Similarly, Dianzani (1969) reported that NDV-infected mouse cells synthesize interferon specific messenger RNA in the presence of protein synthesis inhibitors. Under these conditions, viral replicative events were prevented, thereby indicating that input parental viral RNA or viral protein(s), or both, were the inducer for the synthesis of interferon specific messenger RNA.

The information available at present does not resolve whether single stranded RNA is sufficient to induce interferon production or double stranded RNA is the necessary form. In addition, the role of viral protein(s) in the process of induction is not well understood.

MATERIALS AND METHODS

Materials

Virus Strains

Vaccinia virus (VV). The N. Y. 914 strain, isolated by Dr. G. E. Gifford from commercial lymph vaccine, was employed.

Semliki Forest virus (SFV). Kumba strain was obtained from Dr. J. Porterfield, National Institute for Medical Research, London, England.

Vesicular stomatitis virus (VSV). Indiana strain was kindly supplied by Dr. Samuel Baron, National Institute of Health, Bethesda, Maryland.

Newcastle disease virus (NDV). Hertz strain was also obtained from Dr. J. Porterfield, The Roakin strain was obtained from the Research Reference Reagent Branch of the National Institute of Allergy and Infectious Diseases and the Cincinnati strain was received from Mr. M. Fruitstone, Department of Microbiology, University of Florida.

Cell Cultures

Primary chick embryo cell cultures. Chick embryo (CE) cell cultures were prepared as described in Methods.

Mouse L cell cultures. The continuous L cells (strain 929) were

kindly supplied by Mr. M. Fruitstone.

Media

Balanced salt solution. Gey's balanced salt solution (BSS) was employed in the growth and maintenance media for CE cell cultures.

Growth medium. The growth medium for CE cell cultures consisted of BSS, 5% calf serum, 0.1% sodium bicarbonate, 0.1% lactalbumin hydrolysate (Nutritional Biochemicals) and 0.1% proteose peptone (Difco). For L cell cultures, Eagle's minimum essential medium (MEM) with 10% calf serum was used.

Maintenance medium. The maintenance medium (MM) for CE cell cultures consisted of Gey's BSS with 0.1% lactalbumin hydrolysate, 0.1% proteose peptone, 0.1% yeast extract (Difco "yeastolate") and about 0.1% sodium bicarbonate. For determination of RNA synthesis in cell cultures, a modified maintenance medium (MMM) was employed in which yeast extract was omitted and 2.5% calf serum was added in some cases.

Overlay medium. The overlay medium used for plaque assay consisted of either 1% methyl cellulose in MEM with 10% calf serum, or chick embryo cell culture growth medium containing 5% calf serum and 0.5% agar (Ion agar No. 2, "Oxoid" division of Oxo, Limited).

Reagents

Rabbit reticulocyte buffer (RSB). RSB was prepared according to Penman et al. (1963). The composition of this buffer was 0.01 Tris-(hydroxymethyl)aminomethane(Tris), 0.01 M potassium chloride and

0.0015 M magnesium chloride with pH adjusted to 7.2 by the addition of 0.5 M hydrochloric acid.

Phosphate-chloride buffer. This buffer was made as described by Petermann and Pavlovic (1963) and consisted of 0.001 M potassium phosphate and 0.005 M magnesium chloride, pH 6.8.

Acetate buffer. This buffer was prepared according to the method of Friedman (1968) and consisted of 0.1 M sodium chloride, 0.01 M sodium acetate and 0.0005 M magnesium chloride with pH adjusted to 5.1 by addition of 0.5 M hydrochloric acid.

Sucrose solutions. Ribonuclease-free sucrose (Mann Research Laboratories, New York) was employed for all the sucrose solutions. The following sucrose solutions were used:

a) 1.8 M sucrose solution in RSB

b) 0.5 M sucrose solution in RSB

c) 0.3 M sucrose solution in phosphate--chloride buffer

d) 25% (w/v) and 8% (w/v) solutions of sucrose in RSB, pH 7.2

e) 30% (w/v) and 15% (w/v) sucrose solutions in 0.1 M potassium chloride, 0.01 M tris(hydroxymethyl)aminomethane (Tris) and 0.001 M ethylendiaminetetracetate (EDTA) buffer, pH 7.1

f) 25% (w/v) and 8% (w/v) sucrose solutions in 0.01 M tris, 0.4 M sodium chloride buffer, pH 7.2. In some experiments, the molarity of sodium chloride was varied at 0.2, 0.3, 0.4 and 0.6 M in tris buffer, sucrose solutions.

Ribonuclease solution. Ribonuclease A (Worthington Biochemical Corporation, Freehold, New Jersey) was dissolved in 0.1 M KCl, 0.01 M Tris, 0.001 M EDTA buffer, pH 7.1, to a final concentration of 20 µ g/ml.

Antibiotics. To all media was added 250 units/ml of potassium penicillin G and 100 µ g/ml of streptomycin sulphate.

Radioisotope. Uridine-5-H^3 was obtained from New England Nuclear Corporation, Boston, Mass. The specific activity of the isotope preparations were either 7 C/mM or 28.1 C/mM.

Scintillation fluid. The radioactive samples were diluted in scintillation fluid containing 4 g of 2, 5-bis-2-(5-tert-Butylbenzoxazolyl) thiophene (BBOT) (scintillation grade, Packard), 500 ml toluene (Fisher Scientific Co.) and 500 ml of methanol (Fisher Scientific Company).

Sodium deoxycholate solution. A stock solution of 10% (w/v) sodium deoxycholate was prepared in acetate buffer, pH 5.1.

Phenol. Phenol (Fisher Scientific Co.) was distilled and hydroxyquinoline was added to a concentration of 0.1%. The distilled phenol was saturated with 10 x acetate buffer, pH 5.1, before use.

Bentonite suspension. Bentonite powder (Fisher Scientific Co.) was processed according to the method of Petermann and Pavlovic (1963). The bentonite concentration in coarse suspension was 73 mg/ml.

Actinomycin D. This reagent was a gift from Merck, Sharpe and Dohme, Rahway, New Jersey. Stock solution contained 100 µg/ml of

actinomycin D and was kept in the dark at -20° C.

Semliki Forest virus antiserum. SFV antiserum was kindly supplied by Dr. S. T. Toy. The antiserum at a final dilution of 1:20 was capable of neutralizing approximately 99% of virus in 60 minutes at 37° C.

Methods

Cell Cultures

Chick embryo cell cultures. The method of Lindenmann and Gifford (1963a) was followed with some modifications. Chick embryos, 10 to 11 days old, were decapitated, eviscerated and washed once with Gey's BSS. The embryos were minced by forcing through a "Luer Lok" syringe into a "Bellco" trypsinizing flask. Trypsinization of the tissues was carried out for 30 minutes at 37°C with continuous stirring in 20 volumes of 0.02% (w/v) trypsin (Grand Island Biological Co., Long Island, New York) in Gey's BSS without calcium and magnesium. Large, undigested tissue fragments were removed by filtering the cell suspension through a gauze filter. The trypsin was removed by centrifuging the cell suspension for 20 minutes at 200xg at 5°C. The cells were resuspended in growth medium and recentrifuged for 20 minutes at 200xg and suspended again in growth medium. The cells were then passed through a coarse sintered glass filter using negative pressure and dispensed either at a concentration of 12×10^6 cells in a volume of 5 ml into plaque bottles (2-ounce, screw-capped, square, soft glass bottles having a rectangular side of 3 x 6

cm) or 1 x 10^8 cells into 32-ounce glass prescription bottles (rectangular side 17 x 7 cm). A cell monolayer was usually formed within 24-48 hours of incubation at 37°C.

Mouse L cells (Strain 929). The cell cultures were maintained and propagated in Eagle's MEM with 10% calf serum. The cell monolayers were maintained in 32-ounce bottles. For passaging the cells, the growth medium was decanted and the cell monolayer was washed with Gey's BSS and 10 ml of 0.02% (w/v) trypsin was used to dislodge the cells from glass surface. After an incubation period of 10 to 15 minutes at room temperature, the cells were removed from the glass and counted in a hemacytometer chamber. To form a monolayer, 2-ounce plaque bottles received 1-2 x 10^6 cells and 32-ounce bottles were plated with 8-10 x 10^6 cells.

Growth, Purification and Assay of Viruses

Vaccinia virus

Preparation. Vaccinia virus was propagated in 11-to 12-day-old chick embryos by inoculating 200 plaque forming units (pfu) of virus in 0.2 ml volume on the chorio-allantoic membrane and incubating the eggs at 37°C for 46 to 48 hours. The eggs were chilled at 5°C for several hours, the infected membranes were removed, frozen at -60°C and homogenized with a mortar and pestle with sterile carborundum employing MM as a diluent. The suspension was then centrifuged at 800xg for 30 minutes to remove coarse particles. The supernatent fluid was dispensed into glass ampules which were sealed

and stored at -60°C.

Assay. Vaccinia virus was titrated by the method of Lindenmann and Gifford (1963a). Growth medium was decanted from the 2-ounce plaque bottles and different dilutions of virus in maintenance medium were dispensed in a volume of 2 ml per bottle. Normally, four bottles were employed for each dilution of virus tested. The bottles were incubated undisturbed on a flat, level surface at 37°C for 46 to 48 hours. After the incubation period, the medium was decanted from the bottles and the monolayers were stained with 0.1% crystal violet for 3 to 5 minutes, washed several times in running tap water and inverted to dry. The plaque were counted after enlarging the monolayer's image 6 to 7 times with a photographic enlarger.

Semliki Forest virus

Preparation. Semliki Forest virus was propagated in the brains of 24-to 48-hour-old mice. The newborn ICR strain of mice were intracerebrally inoculated with 0.02 ml of stock virus suspension diluted 1:100 in maintenance medium. The infected brains were harvested 24 hours after inoculation and a 10% (w/v) suspension of brains in maintenance medium was made using a tissue homogenizer with a teflon pestle. The suspension was centrifuged at 800xg for 20 minutes to remove the coarser particles. The virus suspension was dispensed in glass ampules which were sealed and stored at -60°C.

Assay. The SFV was assayed on primary chick embryo cell monolayers employing either the agar overlay or methyl cellulose

techniques. The growth medium was decanted from the bottles and virus dilutions in 0.2 ml volume were added to each plaque bottle. The virus was allowed to adsorb for 60 minutes at room temperature with frequent rocking of the bottles to distribute the virus evenly on the monolayers. The bottles were drained and overlaid with 3 ml of 0.5% molten agar in growth medium with 5% calf serum maintained at 42°C. After the agar solidified, the bottles were transferred to a 37°C incubator for 46 to 48 hours. In the methyl cellulose overlay method, each bottle received 4.5 ml of 1% methyl cellulose in MEM with 10% calf serum after the adsorption of virus. Subsequent steps were similar to the agar overlay technique. After the incubation period, the agar or methyl cellulose was decanted gently from the cells and discarded, the bottles were stained, and plaques were counted as in the vaccinia virus assay (vide supra). The virus preparation, assayed either by methyl cellulose or agar overlay method, displayed no significant difference in titer.

Newcastle disease virus

Preparation. Newcastle disease virus was propagated in 11-to 12-day-old embryonated eggs by inoculating 200 pfu in 0.1 ml volume into the allantoic cavity. After 44 to 48 hours of incubation at 37°C, the eggs were chilled and the allantoic fluid collected and centrifuged at 800xg for 15 minutes at 5°C to remove the coarser particles. The virus preparation was stored at -60°C in sealed glass ampules.

Assay. The NDV was titrated either by agar or methyl cellulose

overlay technique using chick embryo cultures. With the agar overlay, the cell cultures were incubated for 46 to 48 hours; and with methyl cellulose, the incubation period was 72 hours. The other details of the assay system are the same as for SFV (vide supra).

Vesicular stomatitis virus

Preparation. Vesicular stomatitis virus was prepared by inoculating the allantoic cavity of 11-to 12-day-old chick embryos with 200 pfu in 0.1 ml. The incubation period was 36 to 40 hours. The other details of production and assay were similar as described above for NDV (vide supra). Cultures were incubated for 48 hours before staining.

Assay for Neutralizing Antibodies Against SFV

A plaque inhibition test was performed for the detection of antibodies against Semliki Forest virus in rabbit antiserum. The method consisted of employing a constant amount of antiserum with several different dilutions of virus. The various dilutions of Semliki Forest virus in 0.5 ml were mixed with 0.5 ml of antiserum and incubated at 37°C for 60 minutes. Thereafter, the virus-antibody complex was centrifuged at 800xg for 15 minutes. The supernatent fluid was used for Semliki Forest virus assay (vide supra). The virus preparation, incubated with normal rabbit serum, served as a control. The batch of Semliki Forest virus antiserum employed neutralized nearly 99% of SFV. For example, it reduced the titer of SFV from 8×10^7 pfu/ml to 9×10^5 pfu/ml.

Production and Assay of Interferon

Chick interferon

Production. The method employed for the production of interferon was that of Ruiz-Gomez and Isaacs (1963a) with some modifications. The growth medium was decanted from 32-ounce, 48-hour-old chick embryo cell monolayers. The monolayers contained nearly 35×10^6 cells per bottle. The cells were infected with SFV at an input multiplicity of about 0.1 pfu/cell, and to each bottle was added 30 ml of maintenance medium. The bottles were usually incubated for 24 hours at 37°C. The medium containing interferon was harvested and heated at 65°C for 30 minutes to inactivate the virus. Some batches of interferon preparations were centrifuged at 120,000xg for 3 hours to remove most of the inactivated virus particles. The interferon preparations were stored at -20°C.

Assay. The procedure followed for the assay of chick interferon was that of Lindenmann and Gifford (1963b). Growth medium was decanted from CE cell monolayers and then various interferon dilutions and vaccinia virus (usually 200 pfu) were dispensed in a final volume of 2 ml per bottle. Control cultures received the same amount of vaccinia virus in MM without interferon. Usually 4 bottles were employed for each dilution of interferon. The cultures were incubated for 46 to 48 hours, stained with crystal violet and plaques enumerated as described in the vaccinia virus assay system (vide supra). The plaque depressing $dose_{50}$, the amount of interferon preparation in microliters which depressed the plaque number to 50% of the control,

was calculated according to the method of Lindenmann and Gifford (1963b).

L cell interferon

Production. The mouse interferon was produced in L cell monolayers in 32-ounce bottles containing 30-35 x 10^6 cells per bottle. The growth medium was removed and cells were infected with NDV at a multiplicity of about 100 pfu/cell. The virus was allowed to adsorb at room temperature for 60 minutes with frequent rocking of the bottles. Thereafter, the cell cultures were washed with Gey's BSS and 50 ml of Eagle's MEM with 10% calf serum was added to each bottle. The cultures were incubated at 37°C for a period of 24 hours. The medium containing interferon was then harvested, pooled and centrifuged at 800xg for 20 minutes to remove cellular debris. The interferon preparation was dialyzed against 100 volumes of 0.001 N HCl buffer at pH 2 for 5 days with two changes of buffer during this period and finally against two changes of Gey's BSS for a further 12- to 24-hours to restore the pH to neutrality. In some experiments, this preparation was centrifuged for 3 hours at 120,000xg to sediment most of the virus particles. The supernatent fluid was distributed into glass ampules, sealed and stored at -20°C.

Assay. The interferon preparations were assayed on 24 hour-old mouse L cell monolayers in 2-ounce plaque bottles. The various dilutions of interferon were made in Eagle's MEM with 10% calf serum. The medium was drained from the bottles and 2 ml of each dilution

was added to each of 4 plaque bottles. After overnight incubation at 37°C, the interferon dilutions were removed and the cell sheets were washed twice with 5 ml of MM. VSV in 0.2 ml volumes containing 100 to 200 pfu was added to each bottle. The VSV assay and calculation of PDD_{50} units have been previously described (page 32).

Heat Inactivation of SFV

The kinetics of inactivation of SFV was studied. The stock SFV suspension was thawed and incubated at 37°C. At various time intervals, an aliquot was removed and frozen at -60°C. All the samples were assayed for their residual virus titer as described under the assay of SFV (vide supra). The titer of partially inactivated virus was not appreciably changed by one freeze and thaw cycle. Figure 1 shows a representative study of the inactivation of SFV at 37°C. The decrease in virus titer with respect to time of incubation at 37°C was exponential. The rate of inactivation of Semliki Forest virus was one $\log_{10}$ (90%) decrease in virus titer per 3.2 hours of incubation at 37°C. When the stock virus, consisting of 10% infected mouse brain suspension, was centrifuged at 15,000xg for 15 minutes before incubation at 37°C, the rate of inactivation was similar. Four lots of stock virus, prepared in a similar manner, were found to be inactivated at a rate of one $\log_{10}$ in 3.2 hours $\pm$10 minutes.

Incorporation of Uridine into RNA by Chick Embryo Cells

Measurement of uridine incorporation into CE cell cultures was achieved by treating the cells at different time periods with ^{3}H-uridine.

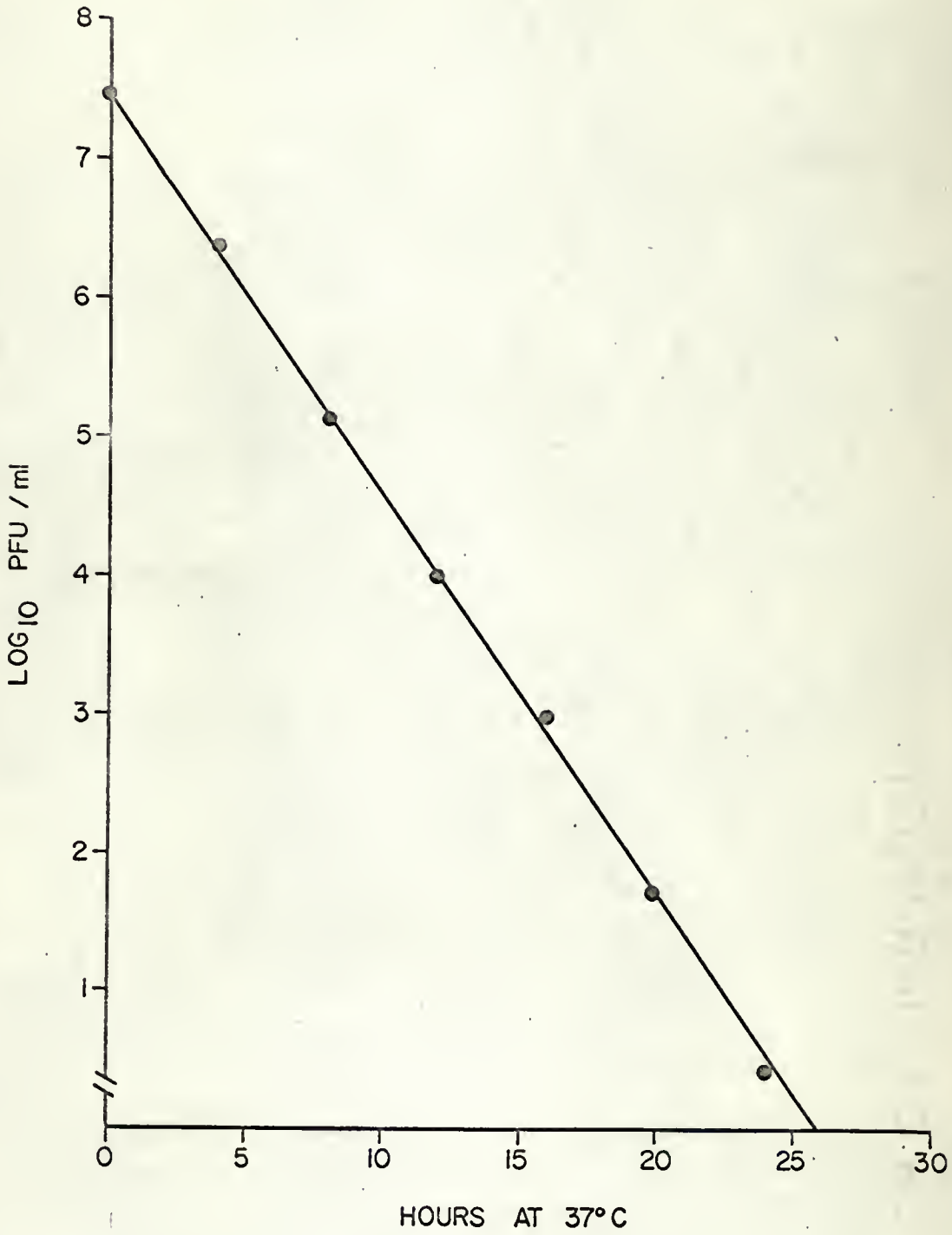

Figure 1. Heat inactivation of Semliki Forest virus at 37°C. Aliquots were removed at various times during incubation and assayed for residual virus.

Plaque bottles, containing approximately 4×10^6 cells, were employed. Growth medium was decanted and the monolayers were washed twice with 2 ml of modified maintenance medium (MMM). The cell cultures after the appropriate treatment (infected or noninfected, with or without actinomycin D, with or without interferon treatment) were supplied with 2 ml of MMM and incubated at 37°C. At different time intervals, 0.3 ml MMM containing 20 μC of ^{3}H-uridine and 3.75×10^{-5} M each of thymidine and deoxycytidine was added to each bottle. Presence of thymidine and deoxycytidine prevented the incorporation of uridine into DNA. The yeast extract was omitted from the maintenance medium because it contains nucleotides which compete with uridine for incorporation into RNA. The cell cultures were replaced in the incubator at 37°C and bottles were gently rocked every 10 minutes to facilitate the even distribution of ^{3}H-uridine on the cell monolayer. To stop the incorporation, 0.1 ml volume of cold uridine (5×10^{-3} M) was added and bottles were immediately placed into an ice bath. The medium was drained from the bottles and the cell monolayers were washed three times with cold 5% perchloric acid (PCA). The cold extraction was done twice more with 5 ml and 10 ml of cold 5% PCA for 5 and 10 minutes respectively. The radioactivity remaining in the last cold PCA extraction was within background levels. Ribonucleic acid was hydrolyzed and extracted in 2 ml of 5% PCA by heating the cell cultures for 30 minutes at 80°C in a water bath.

Radioactivity Measurement

For uridine incorporation studies, 0.2 ml of hot PCA extract

was placed into 20 ml screw-capped, glass vials (Packard) containing 10 ml of scintillation fluid. Samples were counted twice in a Packard "Tri-carb" liquid scintillation spectrometer for 10 minutes and values were expressed as counts per minute.

Uridine Uptake

An experiment was performed to measure the optimum incorporation of uridine into RNA by CE cells employing different concentrations of uridine. The experimental procedure was the same as described above. Figure 2 shows the incorporation of ^{3}H-uridine at various concentrations when a pulse labelling period of 30 minutes was employed. The incorporation of ^{3}H-uridine into cellular RNA increased almost linearly up to about 20 μ C concentrations, but there was no further increase in incorporation when higher concentrations were employed. Uridine incorporation was also determined when the cell cultures were exposed to uridine for different time periods. In this experiment, the cell cultures received 15 μ C of ^{3}H-uridine, but the pulse labelling period was 15, 30, 45 and 60 minutes. Figure 3 shows the results of such an experiment. The rate of incorporation was linear throughout the period of labelling.

An experiment was performed to determine the effect of various concentrations of calf serum in MMM on ^{3}H-uridine incorporation in CE cell cultures. The cell cultures were exposed to 20 μ C for 30 minutes. It is apparent from Figure 4 that incorporation of ^{3}H-uridine into cellular RNA was considerably increased in the presence of

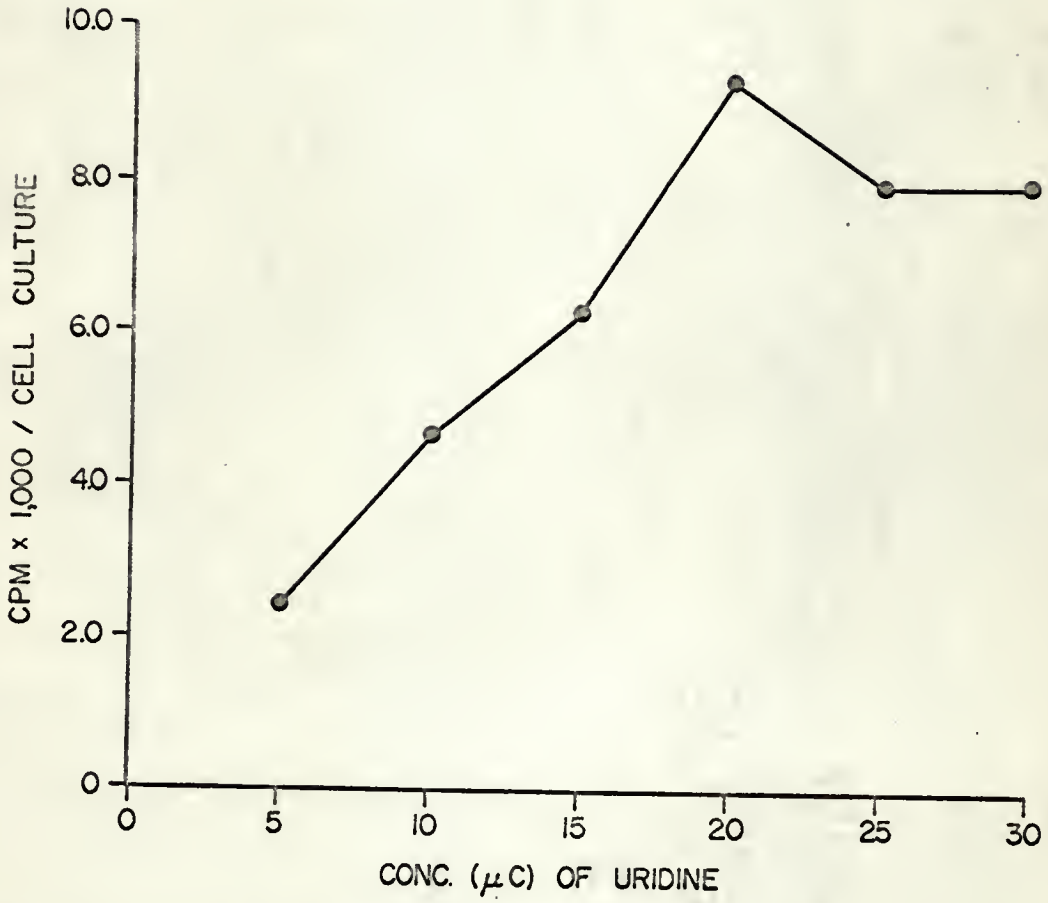

Figure 2. ^{3}H-uridine incorporation in uninfected chick embryo cell cultures exposed to various concentrations of ^{3}H-uridine and incubated at 37°C for 30 minutes.

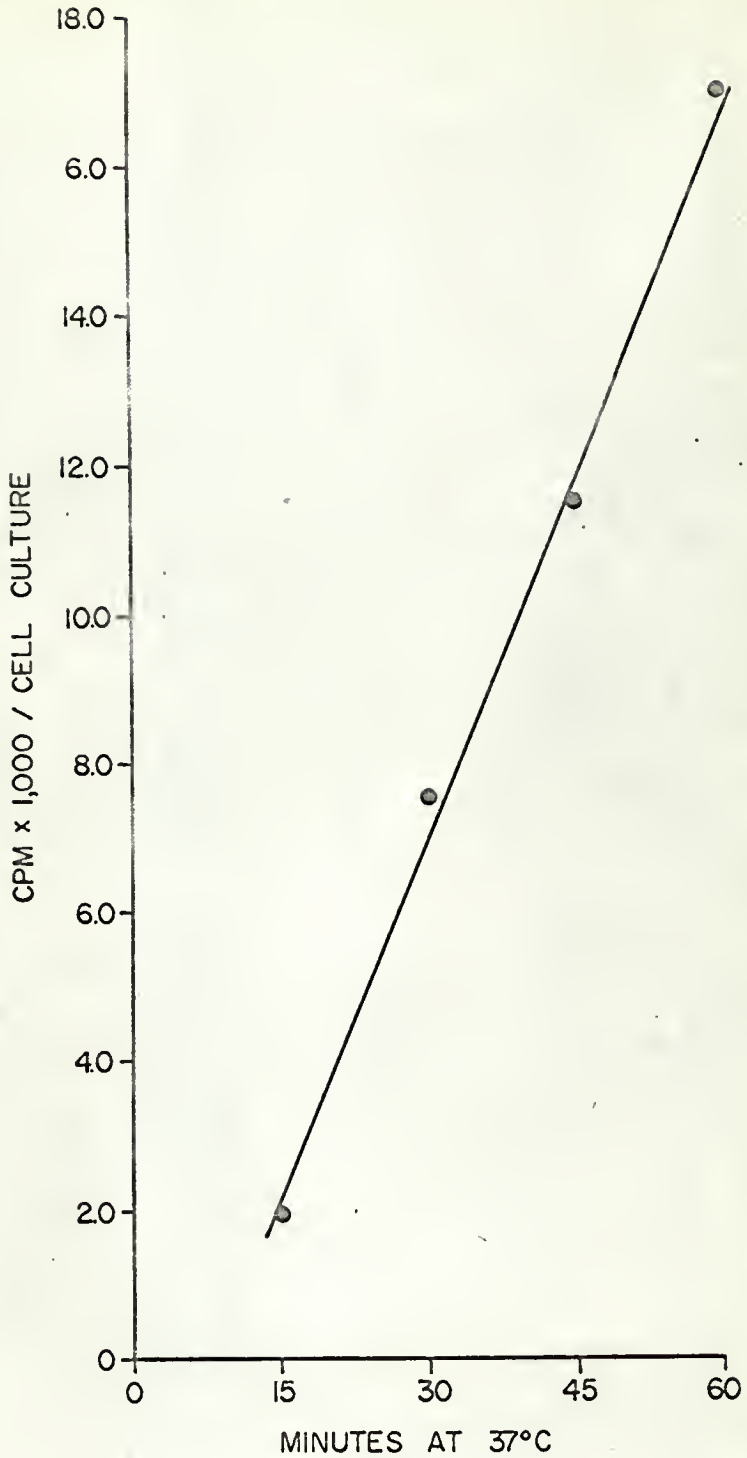

Figure 3. ^{3}H-uridine incorporation in uninfected chick embryo cell cultures exposed to 15 µC of ^{3}H-uridine for various periods of time.

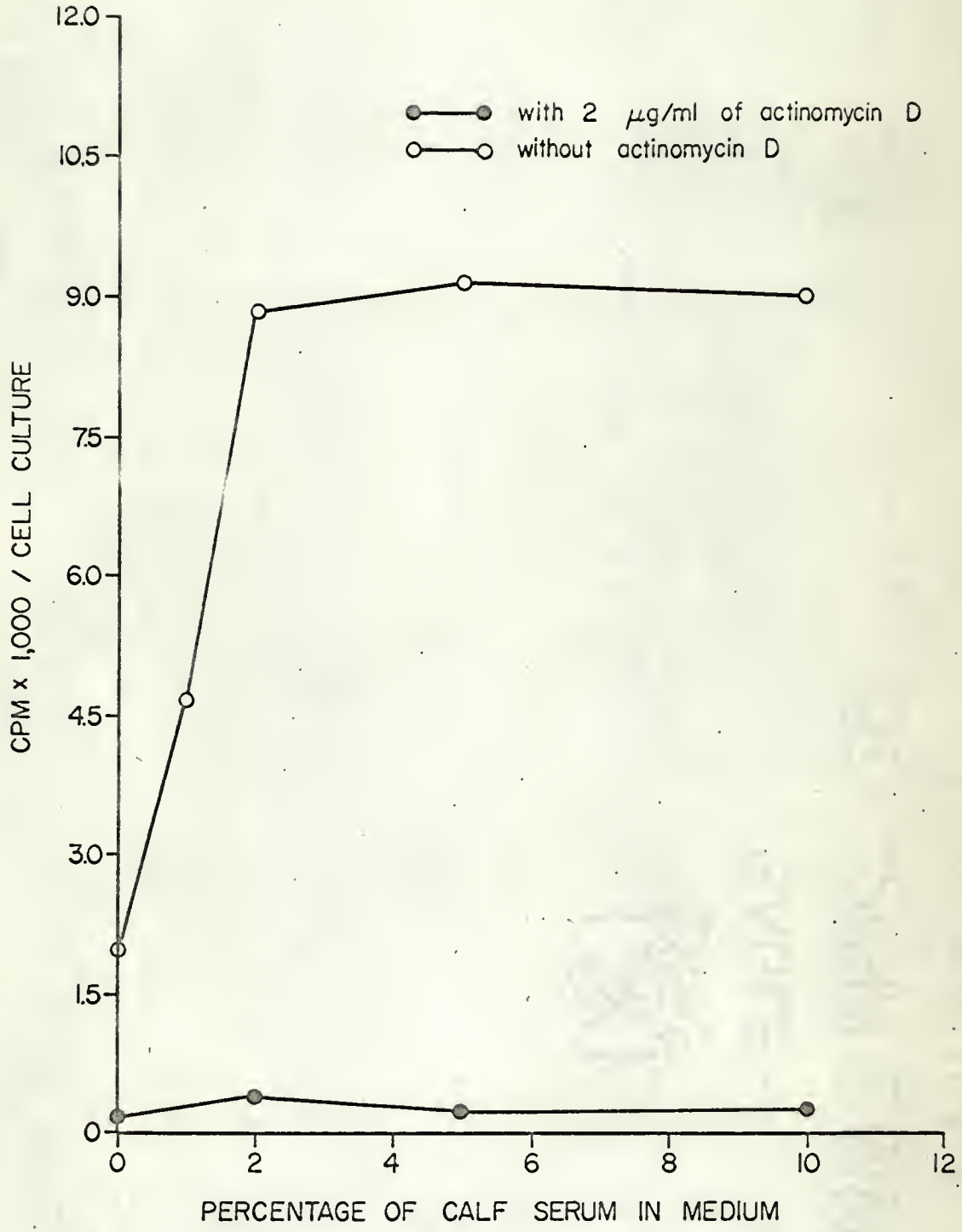

Figure 4. Effect of calf serum on ^{3}H-uridine incorporation of uninfected chick embryo cell cultures with or without actinomycin D. The cell cultures were exposed to 20 μC of ^{3}H-uridine for 30 minutes.

calf serum. The optimum uptake of uridine was found when cell monolayers were maintained in MMM with 2% calf serum. There was no significant increase in uridine incorporation when a higher concentration of calf serum was present in MMM. The data for the uridine incorporation in presence of actinomycin D will be discussed in the next section.

Since in most of the experiments the CE cell monolayers were maintained in MMM for 12 hours or longer, an attempt was made to determine the extent of uridine incorporation into RNA in cell cultures which had been incubated at 37°C for various time periods. As seen in Figure 5, maximum incorporation of uridine occurs when the cells have been incubated for 4 hours. After 6 hours of incubation at 37°C, there was a slight decrease in uridine incorporation at each successive interval of measurement. After 12 hours of incubation, uridine incorporation was reduced by 15 to 20% relative to that at 4 hours.

Inhibition of RNA Synthesis by Actinomycin D in CE Cell Cultures

The effect of actinomycin D on cellular RNA synthesis was determined by measuring ^{3}H-uridine incorporation in CE cell cultures exposed to different concentrations of the drug in 2 ml of MMM. Actinomycin was permitted to remain and RNA synthesis was measured at various intervals. As seen in Figures 6 and 7, actinomycin D concentrations of 0.5 µg/ml or higher inhibited more than 95% of the cellular RNA synthesis. In another experiment, actinomycin D (2 µg) was added to each bottle in 0.1 ml volume with 0.2 ml of MMM.

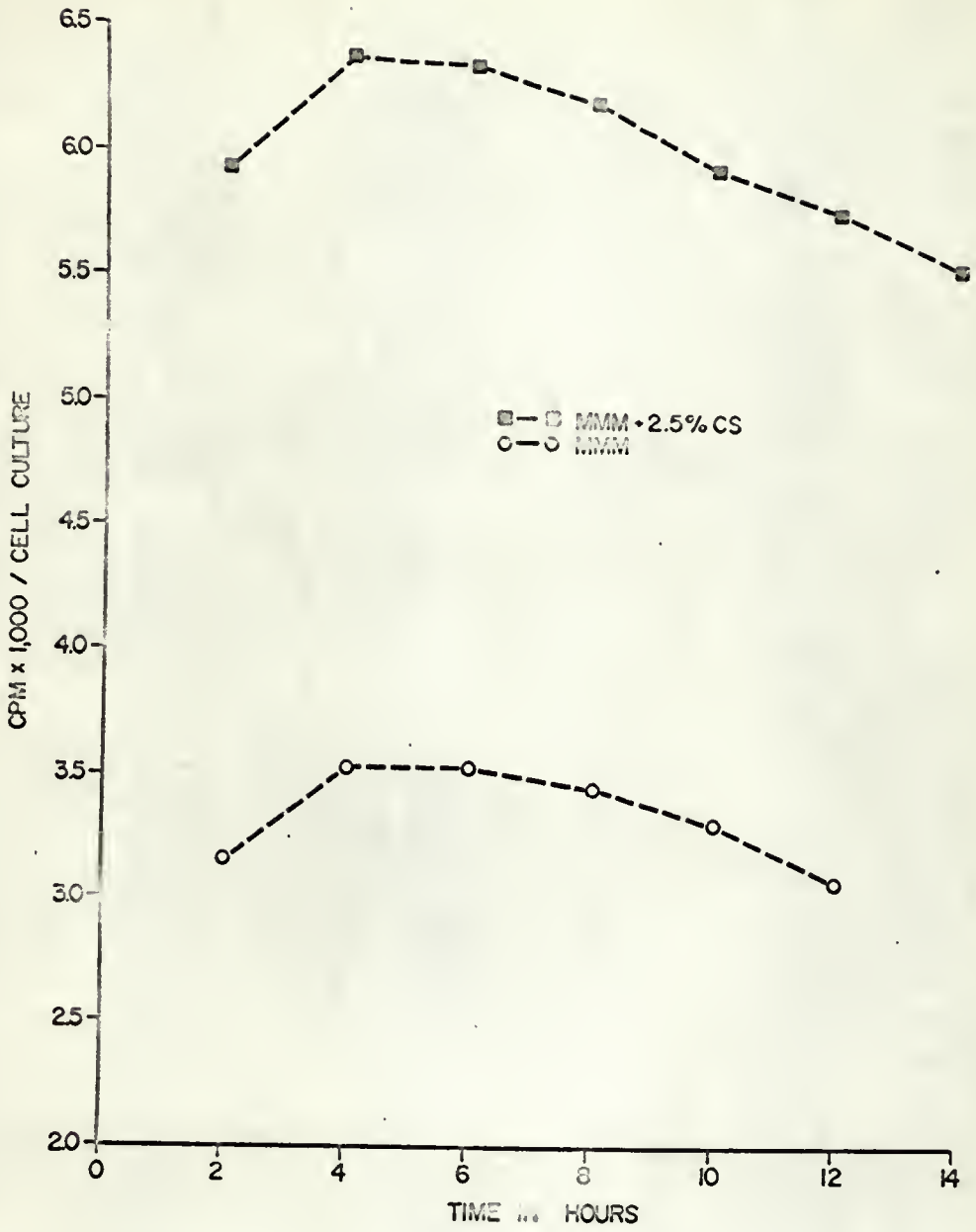

Figure 5. ^{3}H-uridine incorporation, with or without calf (CS) serum in uninfected chick embryo cell cultures. The cell cultures were incubated at 37°C for various periods and then exposed to 20 μC of ^{3}H-uridine for 30 minutes.

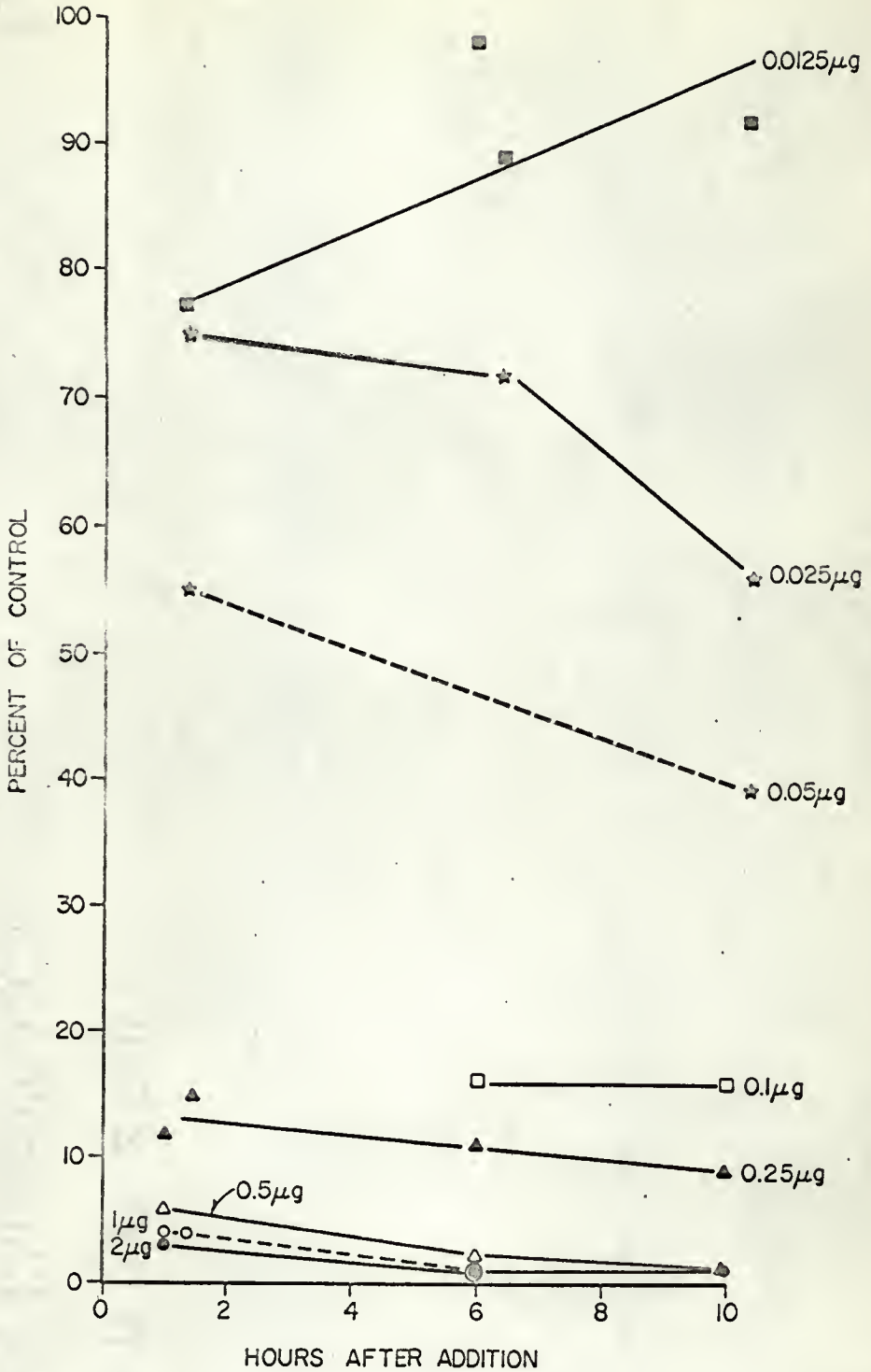

Figure 6. Inhibition of ^{3}H-uridine incorporation in uninfected chick embryo cell cultures with various concentrations of actinomycin D. The cell cultures were exposed to 20 μC of ^{3}H-uridine for 30 minutes at the indicated times.

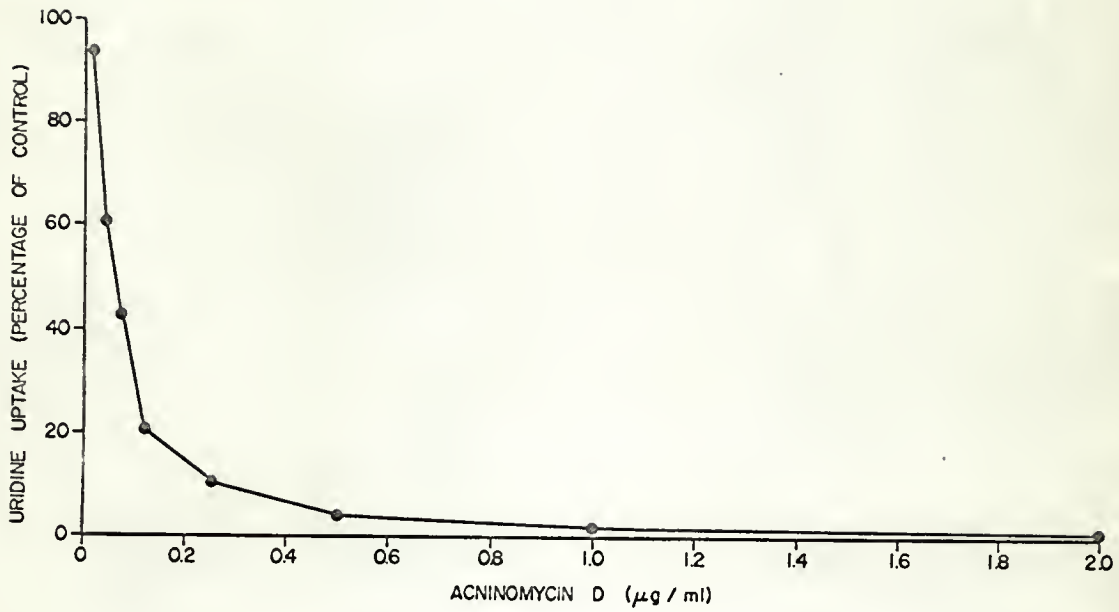

Figure 7. Inhibition of ^{3}H-uridine incorporation in uninfected chick embryo cell cultures with various concentrations of actinomycin D. The values plotted were obtained from Figure 6 at 10 hours after the exposure to actinomycin D.

The bottles were incubated at room temperature for 60 minutes and rocked gently every 10 minutes to ensure even distribution of actinomycin D on cell monolayer. After the incubation period, each bottle was washed twice with MMM and then supplied with 2 ml of MMM per bottle. Further incubation of these bottles was carried out at 37°C. A 30-minute period of labelling with 20 μC of ^{3}H-uridine was made at various times thereafter. Under these conditions, more than 95% of the cellular RNA synthesis was inhibited for nearly 12 hours. However, a gradual recovery of cell monolayers from the effect of actinomycin D was observed after 12 to 15 hours of incubation. In the presence of 2.5% calf serum the uptake of uridine was considerably stimulated, but the percentage inhibition of cellular RNA synthesis by actinomycin D (2 μg/ml) was more than 95% of that of control cell cultures (Figure 4).

Isolation and Purification of Chick Ribosomes

The crude preparation of chick embryo ribosomes was made according to the method of Wettstein, Staehelin and Noll (1963) with several modifications. Ten-day-old chick embryos were decapitated, eviscerated and chilled in crushed ice. All subsequent steps were carried out at 4° to 5° unless otherwise noted. The embryos were forced through a "Luer Lok" syringe. The minced tissue was washed twice with 0.15 M NaCl and twice with RSB. Thereafter, the minced embryos were mixed with 3 volumes of RSB and homogenized with 5 strokes of a motor-driven teflon homogenizer. Under these

conditions, most of the cells were broken, but their nuclei remained intact when examined by light microscopy. The homogenate was centrifuged at 800xg for 10 minutes to remove unbroken cells and other cellular debris. To this supernatant fluid was added 150 mg of coarse bentonite and 10% (w/v) sodium deoxycholate to a final concentration of 0.2%. The suspension was again homogenized with 2-3 strokes and centrifuged at 20,000xg for 15 minutes to remove large particles, such as mitochondria and bentonite. The post-mitochondrial fraction in a 4 ml volume was gently added as a top layer in 10 ml centrifuge tubes previously filled with 3 ml of 0.5 M sucrose in RSB layered over 3 ml of 1.8 M sucrose solution in RSB.

The samples were then centrifuged for 3 hours at 151,000xg in the Beckman Model L centrifuge. The pellets were rinsed and suspended in RS buffer. The suspension was homogenized with 2 to 3 strokes of a motor-driven teflon homogenizer and clarified at low speed (800xg for 10 minutes). This ribosomal preparation was stored at -60°C. The whole procedure, from the death of chick embryos to the freezing step, took 4 to 4.5 hours.

Purification of ribosomes

The purification of ribosomes was achieved by layering 1 to 2 ml of crude preparation on a 26 ml linear, 8 to 25% sucrose gradient. Sucrose gradients were prepared from a standard sucrose gradient maker. The linearity of a typical sucrose gradient, represented by refractive index, is shown in Figure 8. The gradient tubes were

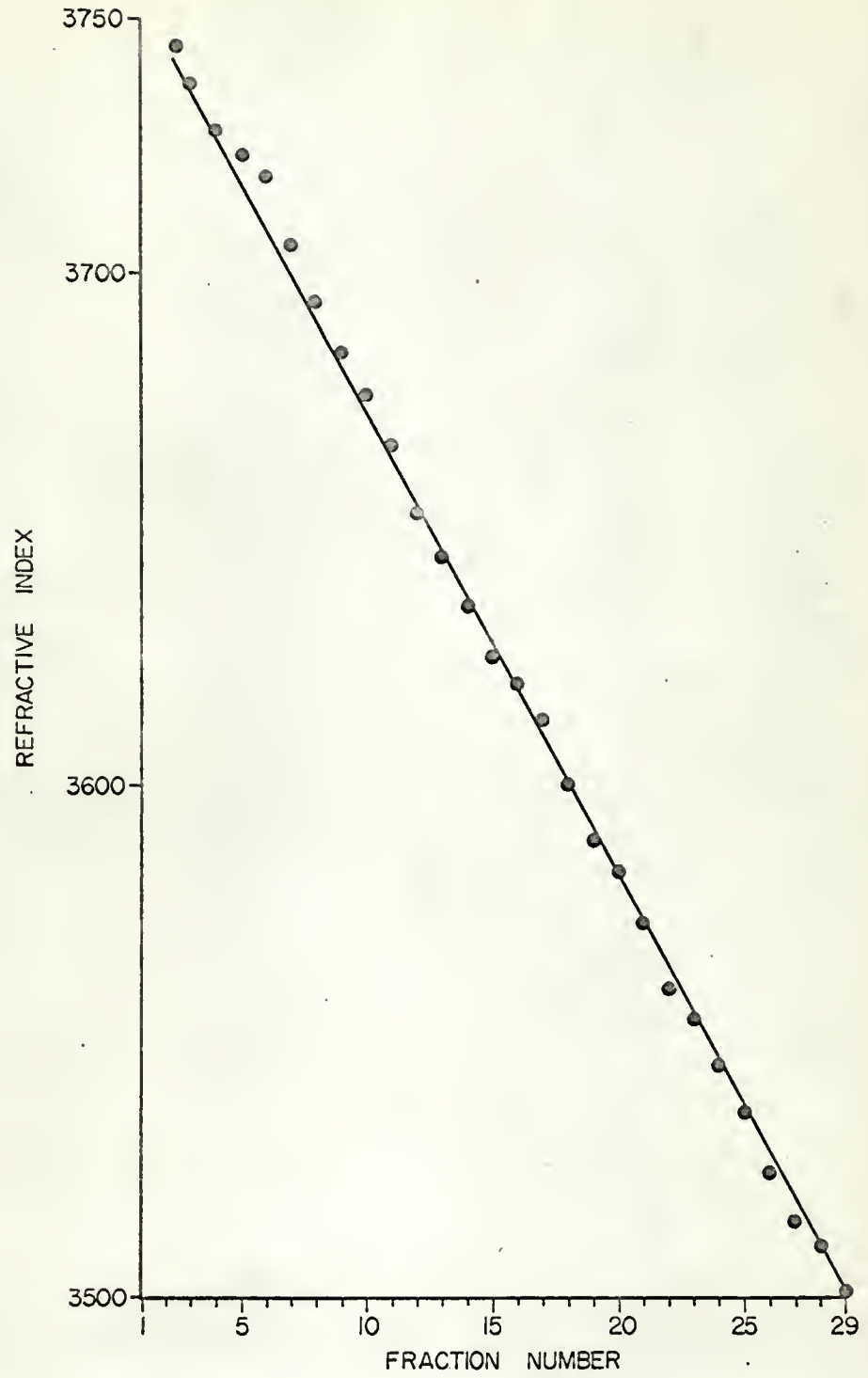

Figure 8. Linearity of sucrose gradient as measured by refractive index of each fraction with an Abbe refractometer.

placed in prechilled swinging buckets of SW 25.1 Beckman rotor and centrifuged at 24,000 rpm for 8 hours. After centrifugation, the bottom of the tube was punctured and 1 ml fractions were by allowing the gradient to drip through a needle. Accuracy of volume in each fraction was achieved by putting mineral oil on the top of the gradient in an air-tight system through a hypodermic needle. Optical density was determined for each fraction with the Beckman DU spectrometer at 258 m μ and in some cases also at 280 m μ. Figure 9 shows a typical profile for these gradients. The ratio of 280:258 was 1:8 as expected for ribosomes. The profile also showed the contamination of crude ribosomal preparation with a heavy and light contaminant which appeared at the bottom and the top of the tube, respectively. Initially, the S values for chick embryo ribosomes were estimated according to the method of O'Brien and Kalf (1967). The crude ribosomal preparation was layered on a 5 to 20% linear sucrose gradient in 0.05 M KCl - 0.005 M $MgCl_2$ - 0.001 M Tris pH 7.6 and centrifuged for 13 hours at 15,000 rpm in the Spinco SW 25.1 rotor. Under these conditions, the chick embryo ribosomal peak was found in a similar position as determined for rat liver ribosomes by O'Brien and Kalf (1967) and was designated 80 S.

Separation of ribosomal subunits

The separation of ribosomal subunits was achieved by the method of Fenwick (1968) with some modifications. The purified chick embryo ribosomes were obtained by the method described above. The

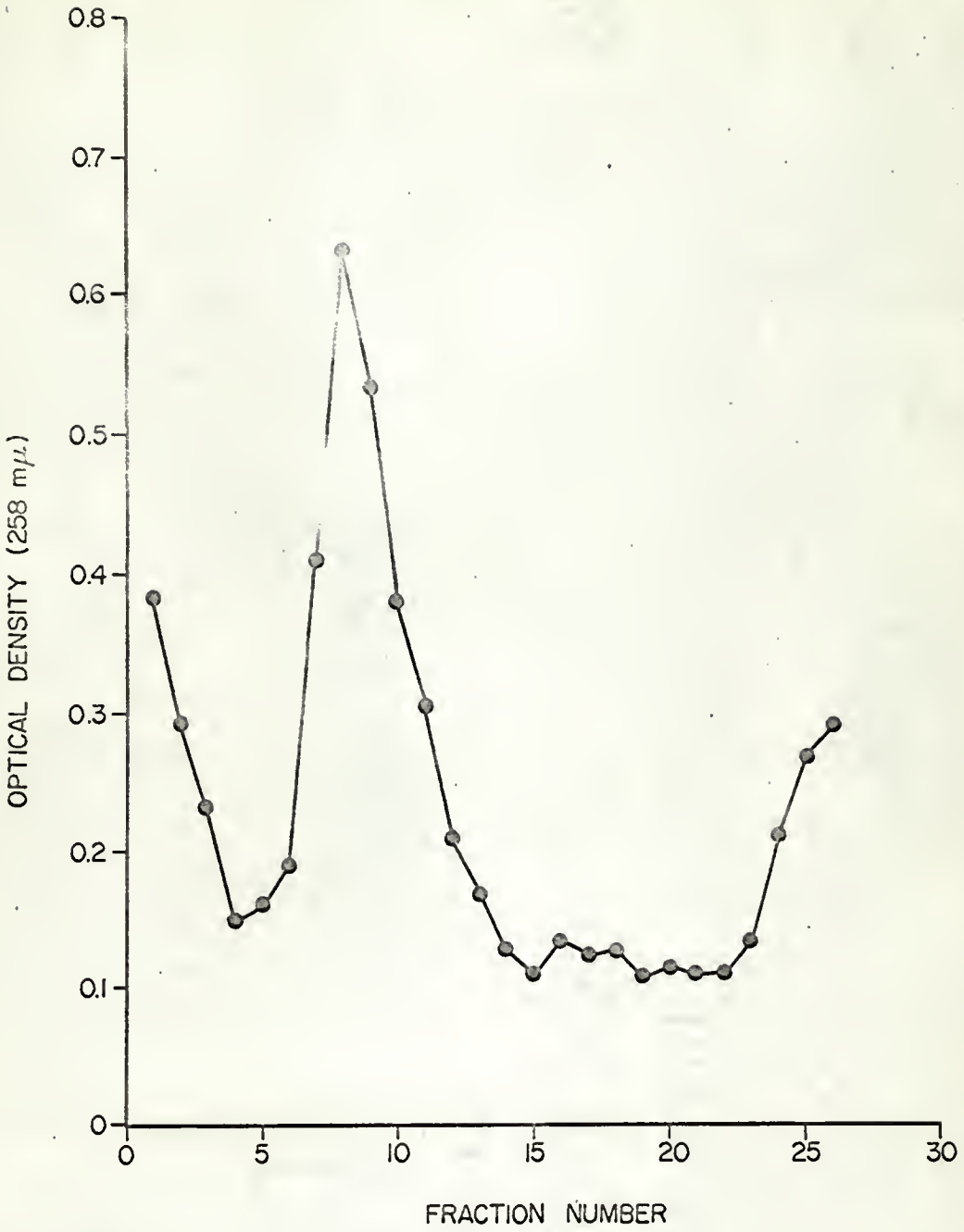

Figure 9. Sucrose gradient analysis of chick embryo ribosomes. One ml of crude ribosomal preparation was layered on 8 to 25% linear sucrose gradient and then centrifuged at 24,000 rpm for 8 hours. The other details have been described under Materials and Methods.

purified preparation was pressure dialyzed against 100 volumes of 0.01 M NaCl - 0.01M Tris HCl, pH 7.2 buffer for 4 hours to reduce the volume and to remove the sucrose. One tenth volume of 4 M NaCl was added to the dialyzed sample and incubated for 5 minutes at 4°C according to the method of Fenwick (1968). One ml of the NaCl treated sample was layered on 26 ml linear gradient of 8 to 25% sucrose and centrifuged for 12 hours at 24, 000 rpm as described above. Figure 10 shows the sedimentation profile of such a gradient. There was very little dissociation of 80 S ribosomes into 60 S and 40 S subunits. The incubation of 4 M NaCl-treated ribosomal preparation for 15 or 30 minutes instead of 5 minutes did not change the sedimentation pattern significantly. In another experiment, the NaCl concentration of ribosomal preparations was raised to 0.2 M, 0.3M, 0.4M or 0.6M. The samples were incubated for 15 minutes at 4°C and were centrifuged in gradients containing the same concentration of NaCl in a buffer (0.01M Tris, pH 7.2) for 12 hours at 24, 000 rpm. Figure 11 shows the sedimentation pattern of ribosomes in such an experiment. The dissociation of chick ribosomes into their subunits was directly proportional to the NaCl concentration. However, a sizeable portion of the ribosomes remained undissociated even in presence of a 0.6 M NaCl concentration. The results were strikingly different from that of Fenwick (1968) who reported complete dissociation of HeLa cell ribosomes into their subunits in presence of 0.2M or more concentration of NaCl. Therefore, it appears that

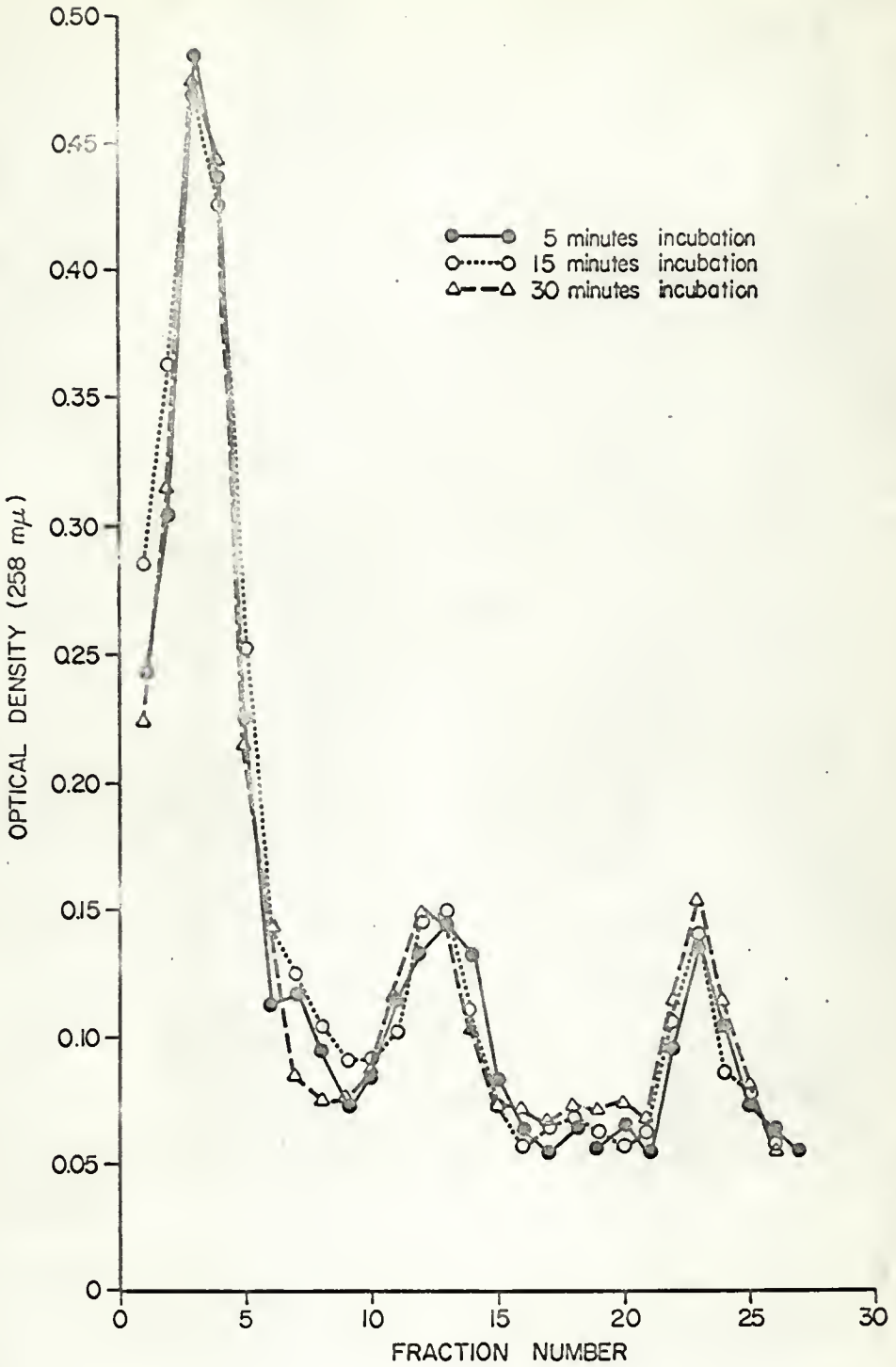

Figure 10. Separation of chick embryo ribosomes into their sub-units. The purified ribosomal preparation was mixed with 1/10th volume of 4 M sodium chloride and incubated at 4°C for 5, 15 or 30 minutes and then centrifuged at 24,000 rpm in a 8 to 25% linear sucrose gradient for 12 hours.

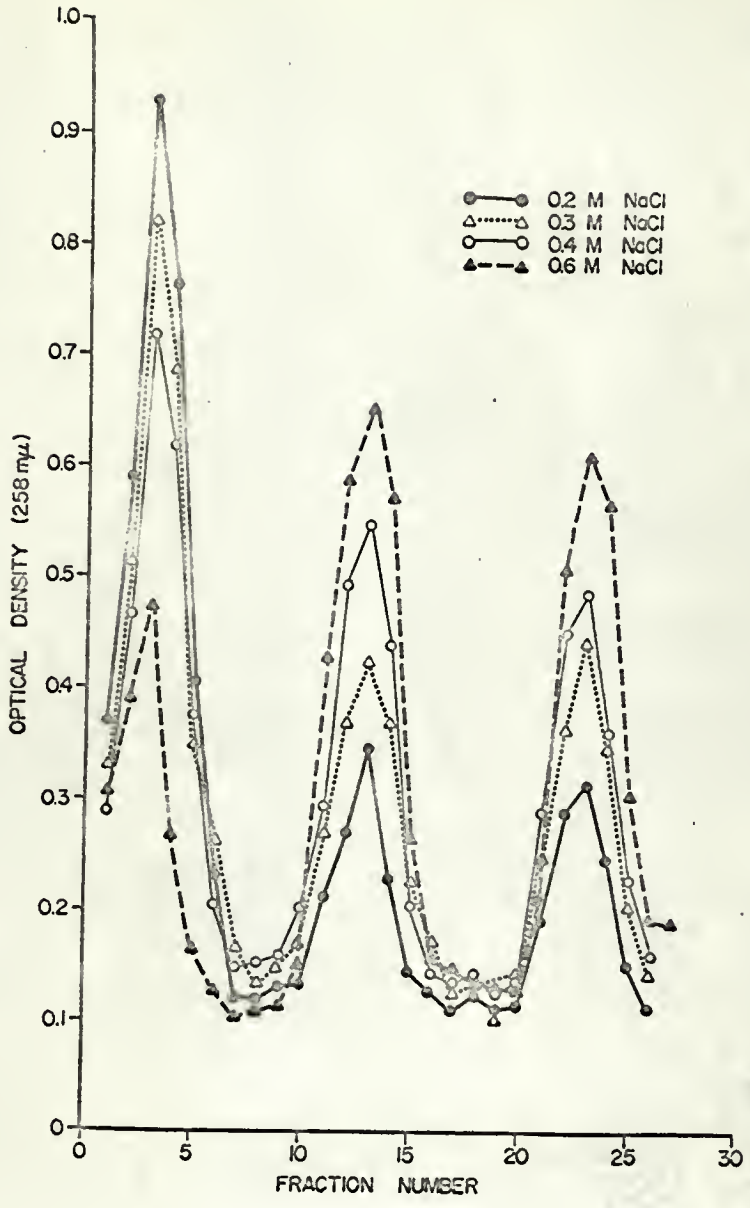

Figure 11. Separation of chick embryo ribosomes into their subunits. The NaCl concentration of ribosomal preparation was raised to 0.2 M, 0.3 M, 0.4 M or 0.6 M and incubated at 4°C for 15 minutes. The samples were then centrifuged in a 8 to 25% sucrose gradient containing the same concentration of NaCl as that of sample and centrifuged at 24,000 rpm for 12 hours.

chick ribosomes were more difficult to dissociate into their component subunits than were those of HeLa cells.

Mouse liver ribosomes. The mouse liver ribosomes were isolated according to the method of Petermann and Pavlovic (1963) with some modifications. Adult, female mice were fasted overnight. They were anesthetized, usually in groups of five, with ether. The livers were removed and chilled in crushed ice. The livers were minced with a scissors and the tissue was forced through a "Luer Lok" syringe. The minced tissue was suspended in 5 volumes of 0.3M sucrose solution and 150 mg of coarse bentonite preparation was added. The suspension was centrifuged at 800xg for 10 minutes and to the supernatant fluid was added 10% (w/v) sodium deoxycholate solution to bring the concentration to 0.2%. The suspension was homogenized with 2 to 3 strokes and centrifuged at 10,000xg for 10 minutes. The pH of the supernatant was rapidly brought to 8 using 0.1N sodium hydroxide, and centrifugation at 20,000xg for 15 minutes was used to remove most of the bentonite. Thereafter, all the steps in mouse liver ribosome isolation and purification were the same as for the chick ribosomes (vide supra).

Detection of Double Stranded, Replicative Intermediate Form of SFV

An attempt was made to detect the double stranded, replicative intermediate form of Semliki Forest virus in CE cell cultures at different times following infection. The method of Friedman (1968) was followed with some modifications. CE cell monolayers, containing nearly 4×10^6 cells, were infected with a multiplicity of

10 pfu/cell of SFV in a volume of 0.2 ml. Each bottle also received 4 μg of actinomycin D in 0.1 ml. The control cells received 4 μg of actinomycin D in 0.1 ml and 0.2 ml of MMM with 2.5% calf serum. The virus was allowed to adsorb for 60 minutes at 4°C, the bottles were rocked every 15 minutes to ensure even distribution of virus. After the incubation period, 2 ml of cold MMM with 2.5% calf serum was added to each bottle and the cell cultures were left overnight at 4°C to synchronize the infection. The following morning, the plaque bottles were transferred to a 37°C incubator for one hour. Then the cell monolayers were washed twice with MMM, supplied with 2 ml of MMM containing 2.5% calf serum for each bottle and returned to the 37°C incubator.

The time when cell cultures were first transferred to the 37°C incubator from 4°C was considered as zero time for viral replication and RNA synthesis cycles.

At various periods of time following the infection, 20 μ C of ^{3}H-uridine in 0.1 ml volume was added to each bottle, and cell monolayers were further incubated for an additional 45 minutes at 37°C. The medium from the cell cultures was then removed, and cells were washed three times with 5 ml of chilled phosphate buffered saline, and twice with 2 ml of 0.1 M NaCl - 0.01 M sodium acetate buffer, pH 5.1. All subsequent steps were carried out in an ice bath unless otherwise stated. To each bottle was added 1 ml of acetate buffer, pH 5.1, and cells were scraped off the glass by the use of

a rubber policeman. Usually cells from 4 bottles of each group (infected with live virus, treated with heat-inactivated virus, or control, untreated cells) were pooled and 15 mg of coarse bentonite suspension and 0.5 ml of 10% SDS solution were added immediately. The RNA extraction was carried out by adding equal volume of distilled phenol and shaking the mixture for 3 to 5 minutes at room temperature. The emulsion was broken by centrifuging at 800xg for 15 minutes and the aqueous layer was separated and extracted again with an equal volume of phenol. Finally, the two phenol phases were mixed and the remaining RNA extracted with 5 ml of acetate buffer, pH 5.1. The aqueous phase of each phenol extraction was combined and centrifuged at 20,000xg for 15 minutes to remove most of the coarse bentonite. Two volumes of cold, absolute ethanol containing 2% potassium acetate were added to the supernatant. The suspension was kept overnight at -20°C and RNA was collected by centrifuging the suspension at 15,000xg for 15 minutes. The precipitate was washed once with 70% ethanol containing 2% potassium acetate, and RNA was again pelleted by centrifuging at 15,000xg for 15 minutes. The RNA precipitate was dissolved in 0.1 M KCl - 0.01 M Tris - 0.001 M EDTA buffer, pH 7.1 and clarified by centrifugation at 10,000xg for 15 minutes. Carrier RNA from L cells was either added during the first phenol extraction period or just before the sucrose gradient analysis of the extracted RNA.

For sucrose gradient analysis, 1 ml (4.927 to 7.126 OD_{260}

units) of extracted RNA was gently layered on a 15 to 30% linear, sucrose gradient prepared in 0.1 M KCl, 0.01 M Tris and 0.001 M EDTA, buffered at pH 7.1. The gradient tubes were placed in pre-chilled buckets of SW 25.1 Beckman-Spinco rotor and centrifuged for 20 hours at 22,000 rpm. After centrifugation, one ml fractions were collected as described under the purification of chick ribosomes. Optical density at 260 mμ was determined for each fraction and 0.1 volume of each fraction was employed for the radioactivity measurements.

Ribonuclease Sensitivity of Viral RNA Isolated from Infected Cells

The viral RNA structure, isolated from CE cells infected with SFV, was tested for its ribonuclease (RNAse) resistance to detect the presence of double stranded replicative forms. The RNAse sensitivity test was performed according to the method of Friedman (1968). RNAse treatment was carried out either before or after the sucrose gradient analysis of RNA extracted from infected cells. In the latter case, the sucrose gradient fractions containing the radioactivity peak were pooled, pressure dialyzed against 100 volumes of 0.1 M KCl, 0.01 M Tris HCl and 0.001 M EDTA, buffered at pH 7.1, for 6 hours to reduce the volume and remove the sucrose from the sample. To 0.9 ml of RNA sample was added 0.1 ml of RNAse solution (20 μ g/ml) in 0.1 M KCl, 0.01 M Tris, and 0.001 M EDTA buffer, pH 7.1, to bring the final concentration of the enzyme to 2 μ g/ml in the reaction mixture, which then was incubated for 10

minutes at 37°C. To stop the RNAse activity, 100 mg of coarse bentonite preparation was added just after the incubation period. The RNA was then immediately extracted with phenol. Carrier RNA (L cell ribosomal RNA) was added, and sucrose gradient analysis was performed as before.

The ribonuclease solution, at the concentration of 2 μ g/ml, was able to completely degrade two OD_{260} units of chick ribosomal RNA when incubated for 10 minutes at 37°C.

RESULTS

Production of Interferon at Different Multiplicities of Infection

The effect of multiplicity of infection of Semliki Forest virus on interferon production was studied. Figure 12 represents the 24-hour yields of interferon in chick embryo cell cultures infected with different input multiplicities of the virus. The best yield of interferon was obtained with an input multiplicity of 0.1 pfu/cell. When the input multiplicity of infection employed was increased to 1 pfu/cell or more, the 24-hour interferon yields were much reduced. The reasons for the multiplicity effect were further studied since it should provide insight into conditions for maximal interferon production as well as possible reasons for the variation in interferon yields.

Production of Interferon as Function of Time Following Infection

To further delineate the differences in interferon yield as a function of multiplicity of infection, an experiment was designed to study the production of interferon in cell cultures at various times after infection with two different multiplicities of Semliki Forest virus. The results are shown in Figure 13. With a multiplicity of infection of 0.1 pfu/cell, there was a significant increase in interferon production beyond 12 hours after infection. In other experiments, with a multiplicity of 0.1 pfu/cell, the increase in interferon production continued beyond 18 hours following infection (e.g., see

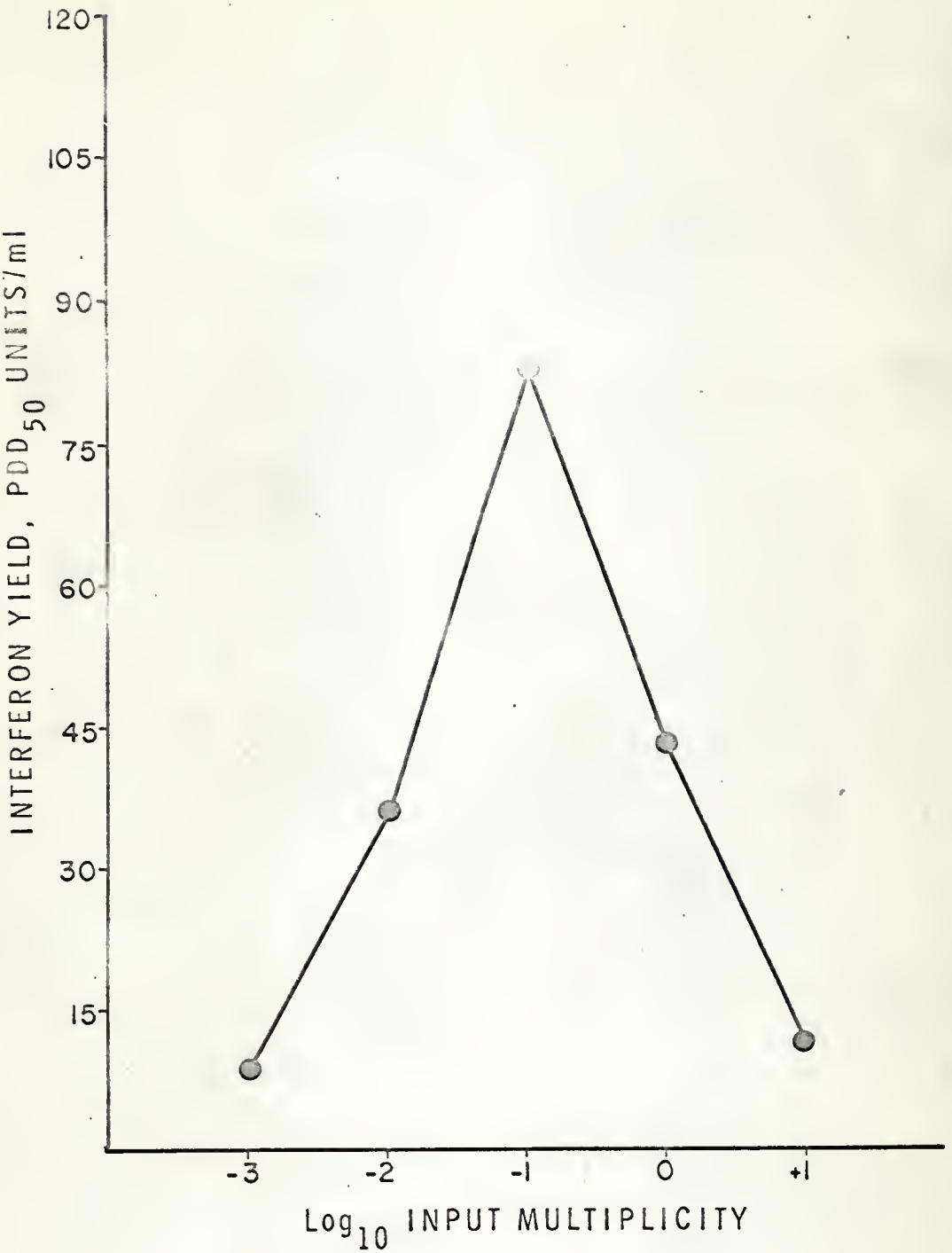

Figure 12. Interferon yields at 24 hours in chick embryo cell cultures infected with various input multiplicities of Semliki Forest virus.

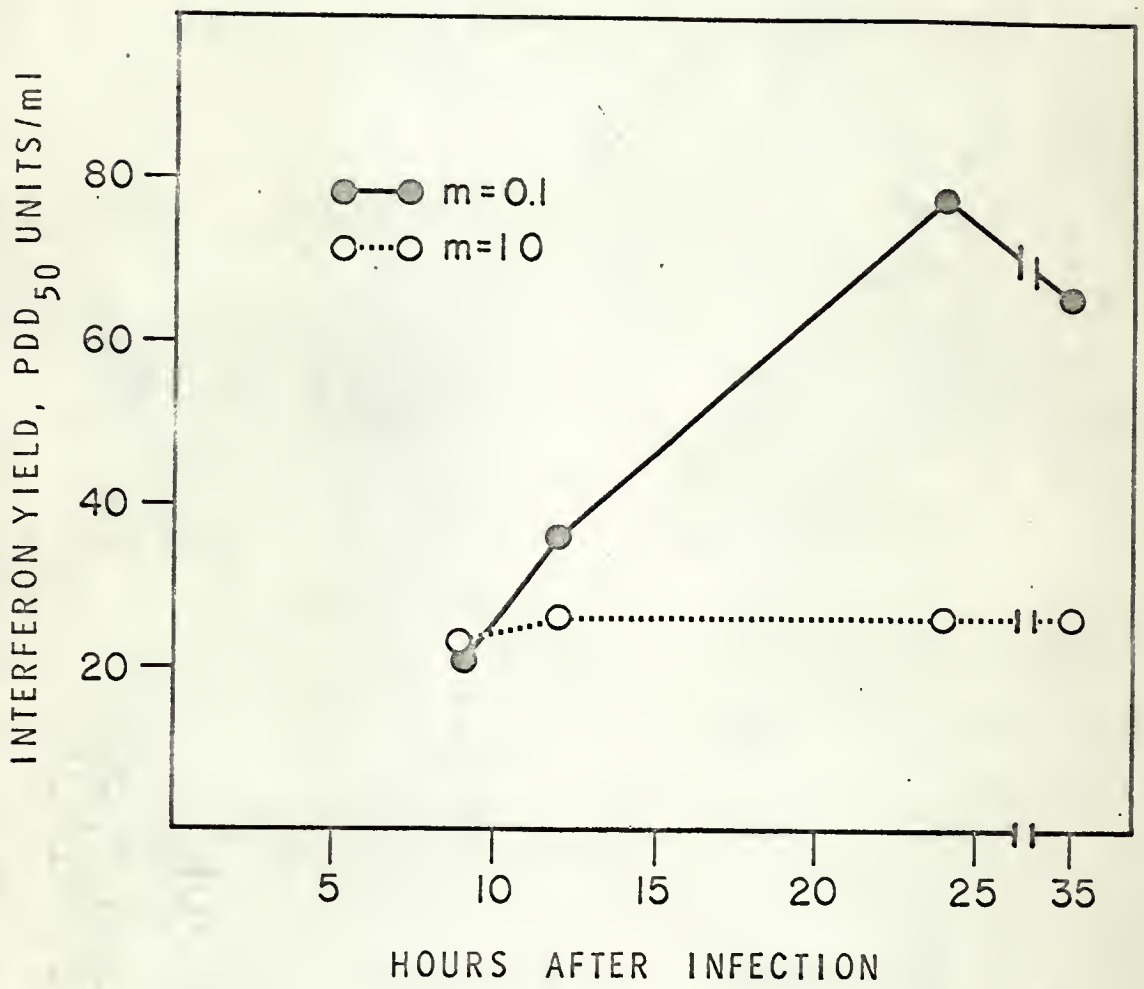

Figure 13. Kinetics of interferon production in chick embryo cell cultures infected with Semliki Forest virus at multiplicities of 10 or 0.1 pfu/cell.

Figure 18). However, the interferon yield was reduced by 36 hours after infection. When cell cultures were infected with 10 pfu/cell of the virus, interferon production nearly ceased by 8 hours after infection (Figure 13 and 17, and Table 2). In this experiment (Figure 13), the 24 hour yield of interferon in cell cultures infected with 10 pfu/cell was nearly 30% of that obtained with 0.1 pfu/cell. This difference usually varied between 14 to 20% in other experiments (Table 2). This data indicated that infection of all the cells (as would be the case with a multiplicity of 10 pfu/cell) resulted in early termination of interferon systhesis. This possibility was further studied.

RNA Synthesis in the Infected Cells

Since interferon production represents the synthesis of induced cellular protein, the viruses which rapidly shut off host macromolecular synthesis would not be expected to be good interferon inducers. Thus, viruses like poliovirus, Mengovirus and vesicular stomatitis virus, which are known to inhibit cellular RNA synthesis (Holland, 1963; Baltimore, Franklin and Callender, 1963; Wagner and Huang, 1966), have also been reported to be poor inducers of interferon in cell cultures (Wagner and Huang, 1966; Burke, 1966). Therefore, the effect of Semliki Forest virus on RNA synthesis of infected cells, at a multiplicity which apparently inhibited interferon production, was studied. Actinomycin D (2 µg/ml) was employed to determine viral specific RNA synthesis in the infected cells. Figure 14 shows RNA synthesis in chick embryo cell monolayers infected with a

TABLE 2

THE EFFECT OF TWO MULTIPLICITIES OF VIRUS ON INTERFERON PRODUCTION

Experiment Number	Multiplicity of Infection (pfu/cell)	Interferon Yield, PDD_{50} Units/ml			
		Hours After Infection			% Inhibition at 10 PFU*
		8	12	24	
1	0.1	21.9	36	78.4	
	10	23.6	25.2	24.8	68.4
2	0.1	12.1	37.2	86.6	
	10	14.3	16	16.2	81.2
3	0.1	9.7	33.2	65.6	
	10	9.2	10.1	9.5	85.5

* Percent inhibition compared to yield of interferon at a multiplicity of 0.1 at 24 hours after infection.

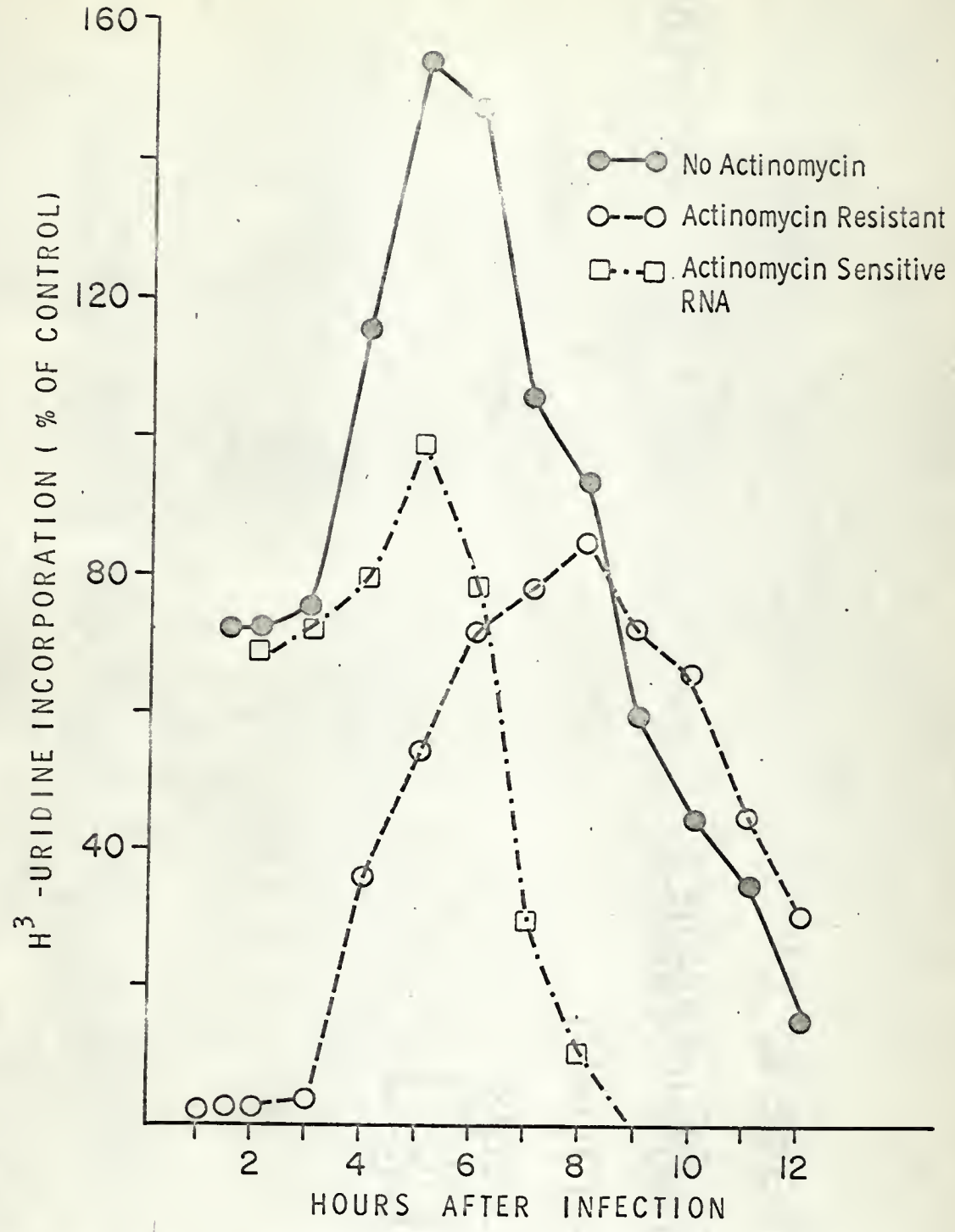

Figure 14. RNA synthesis in chick embryo cell cultures infected with Semliki Forest virus at a multiplicity of 10 pfu/cell with or without actinomycin D. Actinomycin D sensitive RNA was plotted as the difference between total RNA synthesis and actinomycin D resistant RNA synthesis.

multiplicity of 10 pfu/cell of virus with or without actinomycin D. There was an initial increase in total RNA synthesis which reached a peak at 5 hours after infection. In different experiments, this increase varied between 40 to 80% as compared to the control uninfected cells. This early increase was followed by a rapid decline in total RNA synthesis in the infected cells and by 12 hours following infection, it was approximately 14% of that of the control cells. In the presence of actinomycin D, RNA synthesis, presumably of viral origin (referred to as actinomycin resistant RNA synthesis in Figure 14) started after a lag period of 2 to 3 hours and continued increasing until 8 hours following the infection and declined thereafter. The cellular RNA synthesis, plotted as the difference between the total RNA and actinomycin D resistant RNA synthesis at each point, showed a slight increase up to 5 hours after infection in some experiments while this increase was not observed in other experiments. However, cellular RNA (actinomycin sensitive) synthesis in the infected cells was always lower than that of the uninfected cells. After 4 to 5 hours of infection, there was a very rapid inhibition of cellular RNA synthesis and after 8 hours practically all the RNA synthesized was actinomycin D resistant. Thus, interferon synthesis (Figure 13) is terminated at about the time when cellular RNA synthesis ceases almost completely and at the time of maximal viral RNA synthesis.

Growth Curve of Semliki Forest Virus

Replication of Semliki Forest virus was studied to correlate viral specific RNA synthesis with the production of infectious viral

progeny. Figure 15 shows a growth curve of Semliki Forest virus and the accompanying viral specific RNA synthesis in chick embryo cell cultures infected with a multiplicity of 10 pfu/cell. The progeny virus begins to appear after a lag period of 5 hours. Virus maturation occurred between 6 to 10 hours and was essentially completed by 10 hours following infection. The maximal rate of viral specific RNA synthesis was observed at approximately 8 hours following infection. Thus, there was a lag period of 2 to 3 hours between the synthesis of viral specific RNA and the appearance of infectious progeny virus in cell cultures. In the presence of 2 μ g/ml of actinomycin D, which suppressed nearly 95% of cellular RNA synthesis, the virus yield was nearly twice that of cell cultures without actinomycin D. These results confirmed the finding of Taylor (1964) that actinomycin does not inhibit replication of SFV.

The data, so far presented, indicates that in chick embryo cell cultures infected with a multiplicity of 10 pfu/cell, interferon production ceases at the time when progeny virus synthesis was maximal and cellular RNA synthesis was severely inhibited. Thus the cessation of interferon synthesis in the infected cells is most probably due to cell death.

The continued synthesis of interferon beyond 8 hours, when low multiplicity was employed, accounts for the higher yields of interferon obtained. Two possible explanations for this continued synthesis were considered: 1) interferon induction is due to "inactive"

Figure 15. Correlation of viral RNA synthesis with growth of Semliki Forest virus (with or without actinomycin D) in chick embryo cell cultures at a multiplicity of 10 pfu/cell.

virus particles in the virus preparation which do not inhibit host macromolecular synthesis and thereby permit interferon synthesis for longer periods of time, or 2) low multiplicity of infection leads to several cycles of virus infection and, therefore, prolonged interferon synthesis.

Interferon Production by Inactive Virus

The first possibility that interferon production is induced by the nonreplicating virus particles present in Semliki Forest virus preparations was considered. It is not possible to physically separate the noninfectious particles from the infectious particles in Semliki Forest virus preparations. Since Semliki Forest virus is readily inactivated at 37°C, many of the noninfectious particles found may be due to inactivation during production and preparation of the virus stock. Therefore, we chose to increase the number of inactive particles by incubating the virus preparation at 37°C and subsequently determine the effect of such a virus preparation on the induction of interferon synthesis in CE cells. The loss of infectivity followed first order kinetics (Figure 1) and less than 5 pfu/ml remained after 24 hours of incubation.

Figure 16 shows interferon production in chick embryo cells as a function of time after induction. When cells were exposed to inactivated virus, equivalent to 0.1 pfu/cell before inactivation, the 24 hours' yield of interferon was much less than the amount induced by an equivalent amount of live virus. Under these conditions, the

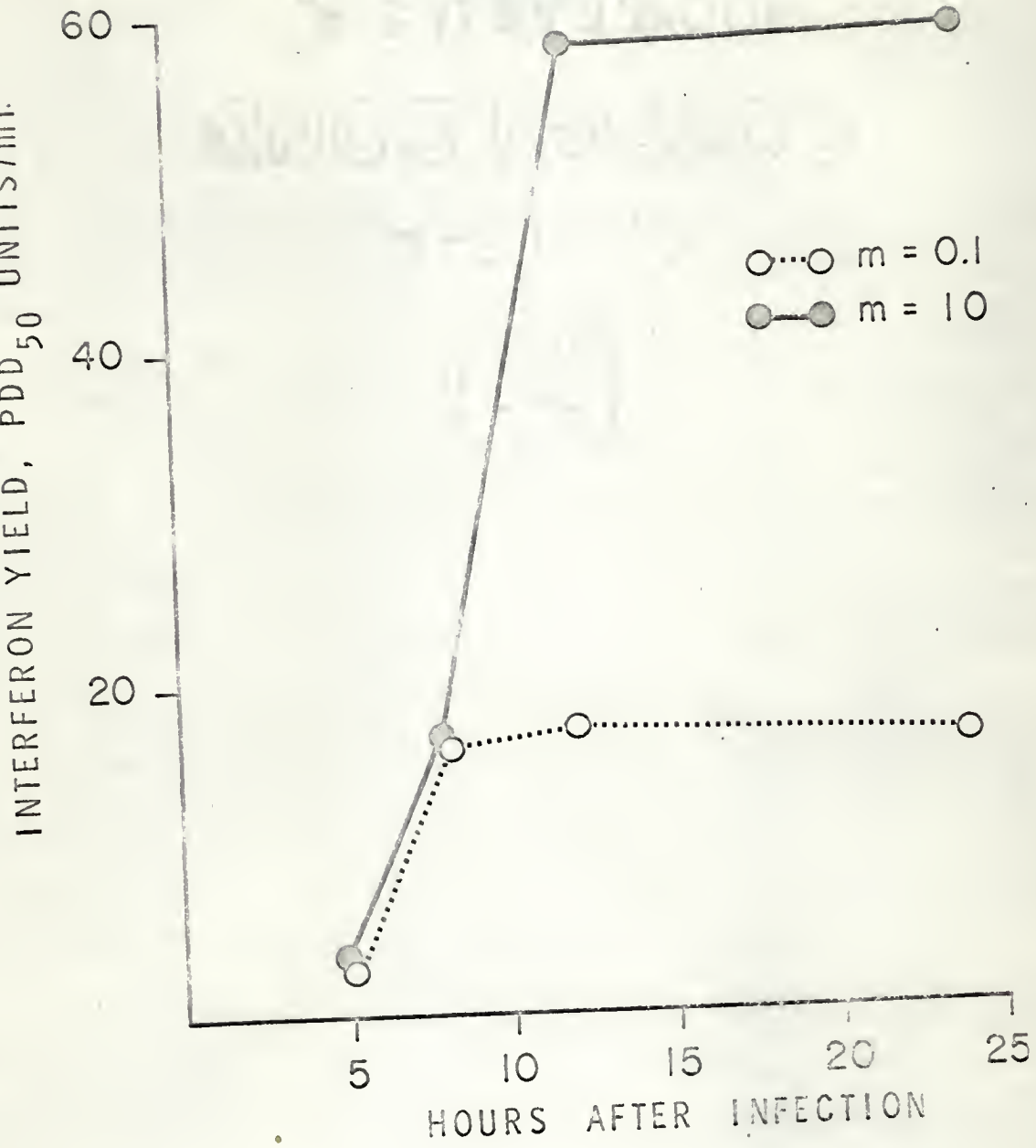

Figure 16. Kinetics of interferon production in chick embryo cell cultures exposed to heat inactivated Semliki Forest virus. The cell cultures received the equivalent of either 10 or 0.1 pfu/cell of the virus.

heat inactivated virus induced small amounts of interferon up to 8 to 12 hours after incubation with no further increase in synthesis thereafter. A higher yield of interferon was obtained when cells were exposed to heat inactivated virus equivalent to 10 pfu/cell. However, the total yield of interferon, in this case, was obtained within 12 hours after the exposure of the cells. Thus the continued synthesis of interferon for 24 hours by cells infected with low multiplicity of the virus is apparently not due to inactive particles present in the virus preparations. However, the data indicates that inactive virus particles do prolong interferon synthesis for some period of time as compared to cells infected with live virus. The kinetics of interferon production in cell cultures exposed to live virus at a multiplicity of 10 pfu/cell or an equivalent amount of the heat inactivated virus were studied (Figure 17). With live virus, interferon synthesis was again nearly completed by 8 hours following infection, while the production of interferon continued for 12 hours in cell cultures induced by heat inactivated Semliki Forest virus. The continued synthesis of interferon for 4 additional hours explains the increased yield of interferon with inactive virus. The maximum yield of interferon with the live virus was nearly 26% of that induced by the heat inactivated virus. Similarly, Burke and Walters (1966) observed that interferon production was essentially completed in 10 hours when chick embryo cells were infected by Semliki Forest virus first at 36°C for 1 hour and then at 42°C. Under these conditions, infectious virus particles were not synthesized but interferon production was, apparently, not affected. The reason for

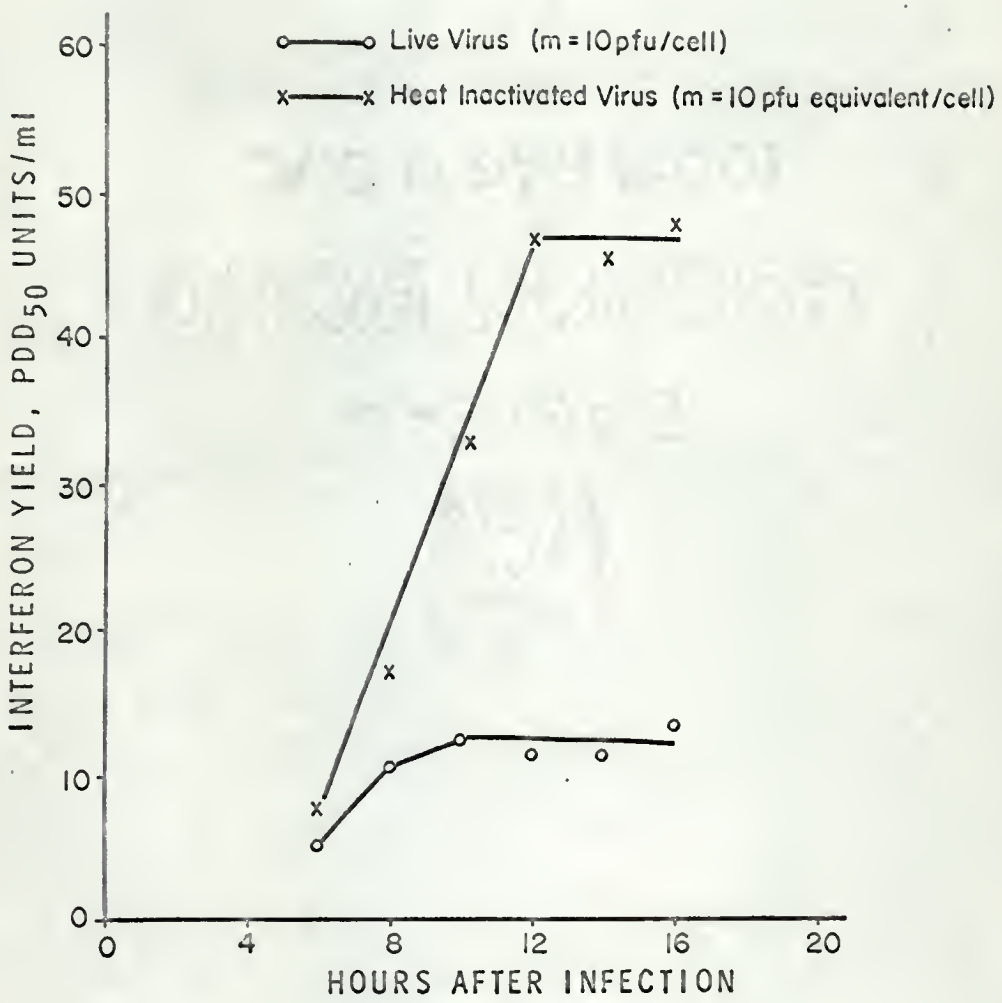

Figure 17. Kinetics of interferon production with heat inactivated and live Semliki Forest virus with cell cultures.

the cessation of interferon synthesis by inactive virus at 12 hours in our study is unknown. In another experiment (Figure 24),it was observed that the heat inactivated virus, equivalent to an original multiplicity of 10 pfu/cell, did not inhibit cellular RNA synthesis of the induced chick embryo cells up to 12 hours after exposure. At 15 hours, cellular RNA synthesis was 86% of the controls and decreased to 69% at 18 hours after exposure (not shown in the figure). The late inhibition could have been due to the replication of the small amount of residual virus in the inactivated virus preparation. Termination of interferon synthesis by 12 hours after exposure to the heat inactivated virus, even though cellular RNA synthesis was apparently normal, conforms to the general observation that interferon synthesis stops at varying times after induction. Cessation of interferon production may be due to the inhibition of interferon production by interferon (Vilcek and Rada, 1962; Cantell and Paucker, 1963; Friedman, 1966b). However, it is difficult to explain why interferon stops its own synthesis in 12 hours in chick embryo cell induced by SFV but does not affect the synthesis for 40 hours in chick embryo cells induced by ultraviolet irradiated influenza virus (Burke, 1966). Alternatively, some cellular control mechanism may be responsible for the termination of interferon production but evidence for such a mechanism is lacking. The cell may also be able to destroy the inducer, thereby preventing the continued induction.

Interferon Production in Presence of Immune Serum

When chick embryo cell cultures are infected with SFV at 0.1

pfu/cell, less than 10% of the cells are initially infected. Since infected cells produce interferon for a maximum of 10 hours, the continuous synthesis of interferon for 24 hours under these conditions is most probably due to the subsequent infection of the remaining cell population by the progeny virus.

To test this possibility, the kinetics of interferon production in chick embryo cell cultures infected with 0.1 pfu/cell of Semliki Forest virus, with or without the addition of SFV antiserum after virus adsorption, were studied. The progeny virus produced by the initially infected cells should be neutralized by the immune serum present and thus would be unable to infect the remaining cells. The cell monolayers were infected at the desired multiplicity in 0.2 ml volume and incubated for one hour at room temperature. After the incubation period, each cell culture was washed twice with 5 ml of Gey's balanced salt solution, supplied with 2 ml of maintenance medium and incubated at 37°C. Following 2.5 hours of incubation, 0.1 ml of immune serum was added to some of the infected cell cultures. Control cell cultures received 0.1 ml of normal rabbit serum. The other details were the same as described under Materials and Methods. Figure 18 shows the results of such an experiment. In presence of the immune serum, the total yield of interferon was obtained within 12 hours of infection. In cell cultures containing normal rabbit serum in place of immune serum, the production of interferon was roughly linear for 24 hours following the infection and was similar to that shown in Figure 13. However,

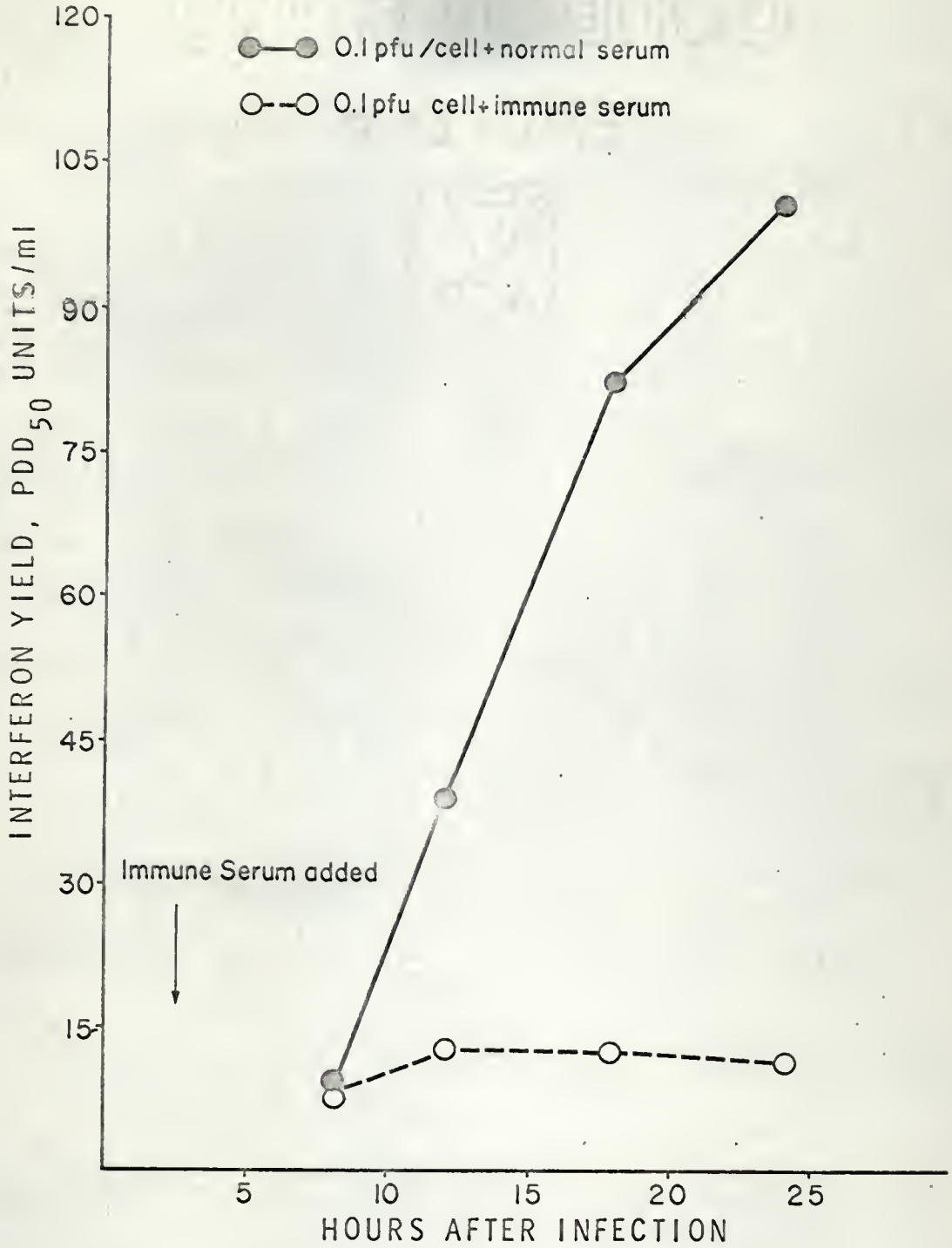

Figure 18. Kinetics of interferon production in presence of immune serum prepared against Semliki Forest virus. Details have been described in the text.

the amount of interferon produced for the first 8 hours was similar in both the cases. The results indicate that continuous interferon synthesis for 24 hours in cells infected with 0.1 pfu/cell multiplicity of the virus is due to the subsequent infection of the cells by the progeny virus.

Interferon Production in Actinomycin D Pretreated Cells

Several investigators have suggested that there are two different mechanisms responsible for the appearance of extracellular interferon following the addition of various inducers. These are, i.e., de novo synthesis versus the release of preformed interferon (Ho and Kono, 1965; Ho, Postic and Ke, 1968; Finkelstein, Bausek and Merigan, 1968). Infection by viruses leads to de novo interferon synthesis in the induced cells (Burke, 1966; Finkelstein, Bausek and Merigan, 1968; Paucker, 1969). Actinomycin D inhibits viral induced interferon synthesis but had no effect on interferon produced in response to endotoxin, poly rI/rC and pyran, a carboxy copolymer (Finkelstein, Bausek and Merigan, 1968) except when very high concentrations were employed. Therefore, interferon production was studied in cells pretreated with actinomycin D to gain information regarding the type of interferon detected following the addition of heat inactivated Semliki Forest virus to cell cultures. The procedure of Gifford and Heller (1963) was essentially followed. Chick embryo cell cultures were exposed to 0.6 μ g/ml of actinomycin D in maintenance medium at 37°C for 4 hours. Thereafter, the maintenance medium was removed and cell cultures were washed twice with fresh

maintenance medium. Control cell cultures were treated in an identical manner except that actinomycin D was not added. The cell cultures were then exposed to the same amount of live or heat inactivated Semliki Forest virus. Other details were the same as described in Materials and Methods.

As shown in Table 3, induction of interferon production by live or heat inactivated Semliki Forest virus in chick embryo cell cultures was almost completely inhibited when the cells were pretreated with actinomycin D. The interpretation placed on these results is that interferon production is induced by the live as well as heat inactivated Semliki Forest virus. Actinomycin D, by selectively inhibiting cellular RNA synthesis, prevented the formation of interferon specific messenger RNA and thus indirectly prevented interferon production.

Viral Specific RNA Synthesis in the Induced Cells

It has been suggested that RNA viruses induce interferon production by synthesizing a double stranded form of RNA which then acts as the inducing agent (Skehel and Burke, 1968b; Hilleman, 1969; Colby and Chamberlin, 1969). Since heat inactivated virus, though unable to replicate but induces more interferon than live virus, the possibility was investigated as to whether heat inactivated Semliki Forest virus undergoes a double stranded, replicative form as a prerequisite for interferon production or if the single stranded viral RNA is in itself the inducing agent. Live virus infection, at a multiplicity of 10 pfu/cell, induces much lower yields of interferon but

TABLE 3

INTERFERON PRODUCTION IN PRESENCE OR ABSENCE OF ACTINOMYCIN D

Virus Preparation	Multiplicity of Infection pfu/cell	Presence or Absence of Actinomycin D	15 Hour Interferon Yields (PDD_{50} Units/ml)
Heat Inactivated Virus	10*	—	64.5
Heat Inactivated Virus	10*	+	3.6
Live Virus	10	—	11.2
Live Virus	10	+	2.0

* Equivalent amount of heat inactivated virus

Note: The virus titer at the end of 15 hours incubation was 6 to 8 x 10^3 pfu/ml in cell cultures exposed to heat inactivated virus.

was employed as a control to detect the double stranded, replicative intermediate form of RNA in the infected cells. Chick embryo cell monolayers were infected with Semliki Forest virus in the presence of 2 μg/ml of actinomycin D. After various periods of incubation at 37°C, 20 μC of ^{3}H-uridine was added for an additional 45 minutes and the RNA was extracted as described in Materials and Methods.

Sucrose gradient analysis of viral specific RNA

The extracted RNA was gently layered on a 15 to 30% linear sucrose gradient and centrifuged at 22,000 rpm for 20 hours in a SW 25 rotor. The gradient fractions were collected in 1 ml aliquots and the optical density measured at 260 mμ and radioactivity measured in the spectrometer. Ribosomal RNA from L cells was employed for the estimation of S value of viral specific RNA. Sucrose gradient analysis of the extracted RNA two hours after infection showed a peak ^{3}H-uridine in the 26 S region with counts extending into ranges of lower S values. When an aliquot of extracted RNA was incubated with ribonuclease (2 μg/ml) for 10 minutes at 37°C before sedimentation, another peak of radioactivity sedimenting in the 20 S region was revealed (Figure 19). When RNA from the infected cells was extracted 4 or 6 hours after the infection, an additional radioactive RNA peak sedimenting at the 45 S region, as shown in Figures 20 and 21, was also obtained. These results are similar to those of Friedman, Levy and Carter (1966) and Sonnabend, Martin and Mecs (1967) who reported that viral specific RNA from the infected cells can be resolved into three components. They also reported that

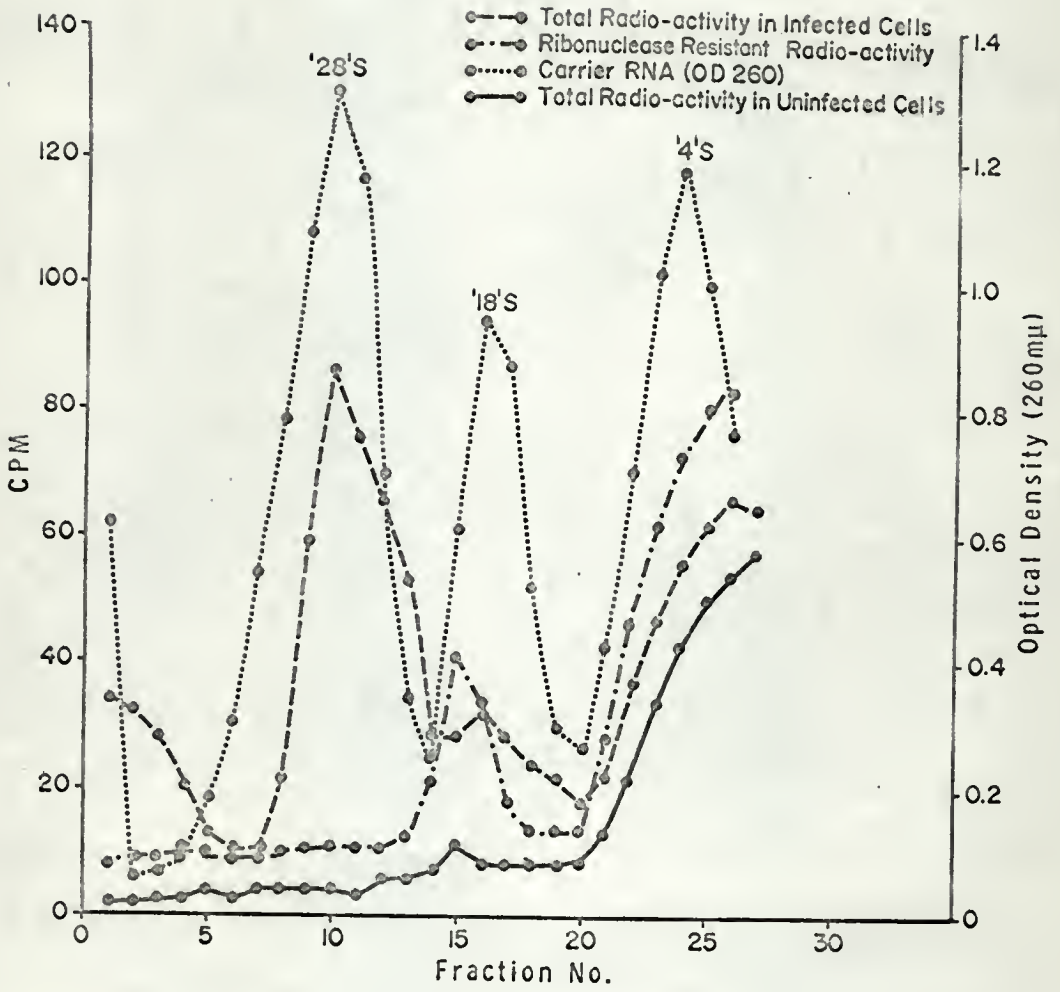

Figure 19. Sucrose gradient analysis of RNA extracted from actinomycin D-treated chick embryo cell cultures infected for 2 hours with Semliki Forest virus. ^{3}H-uridine (20 μC) was added for 45 minutes, and RNA was extracted. L cell RNA was employed as carrier RNA.

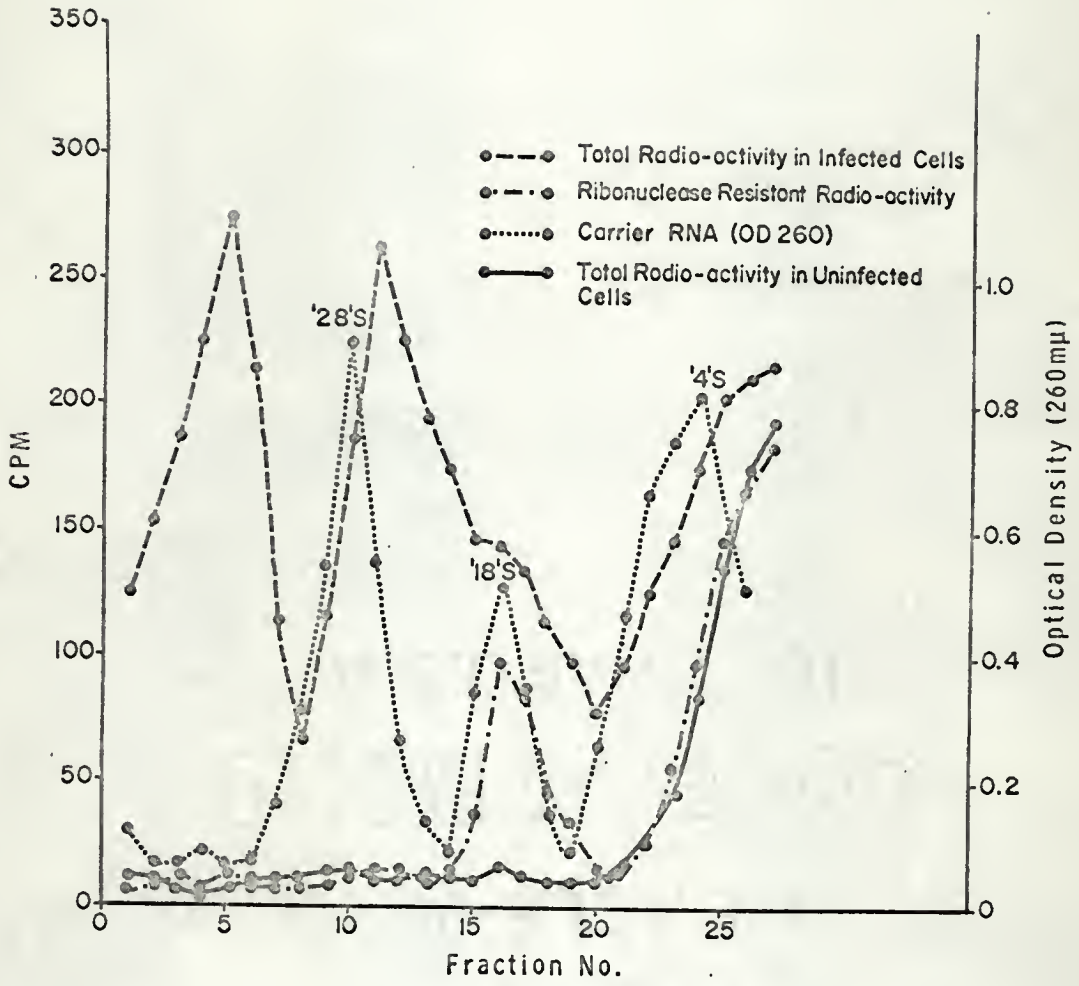

Figure 20. Sucrose gradient analysis of RNA extracted from actinomycin D-treated chick embryo cell cultures infected for 4 hours with Semliki Forest virus. ^{3}H-uridine (20 μC) was added for 45 minutes, and RNA was then extracted. L cell RNA was employed as carrier RNA.

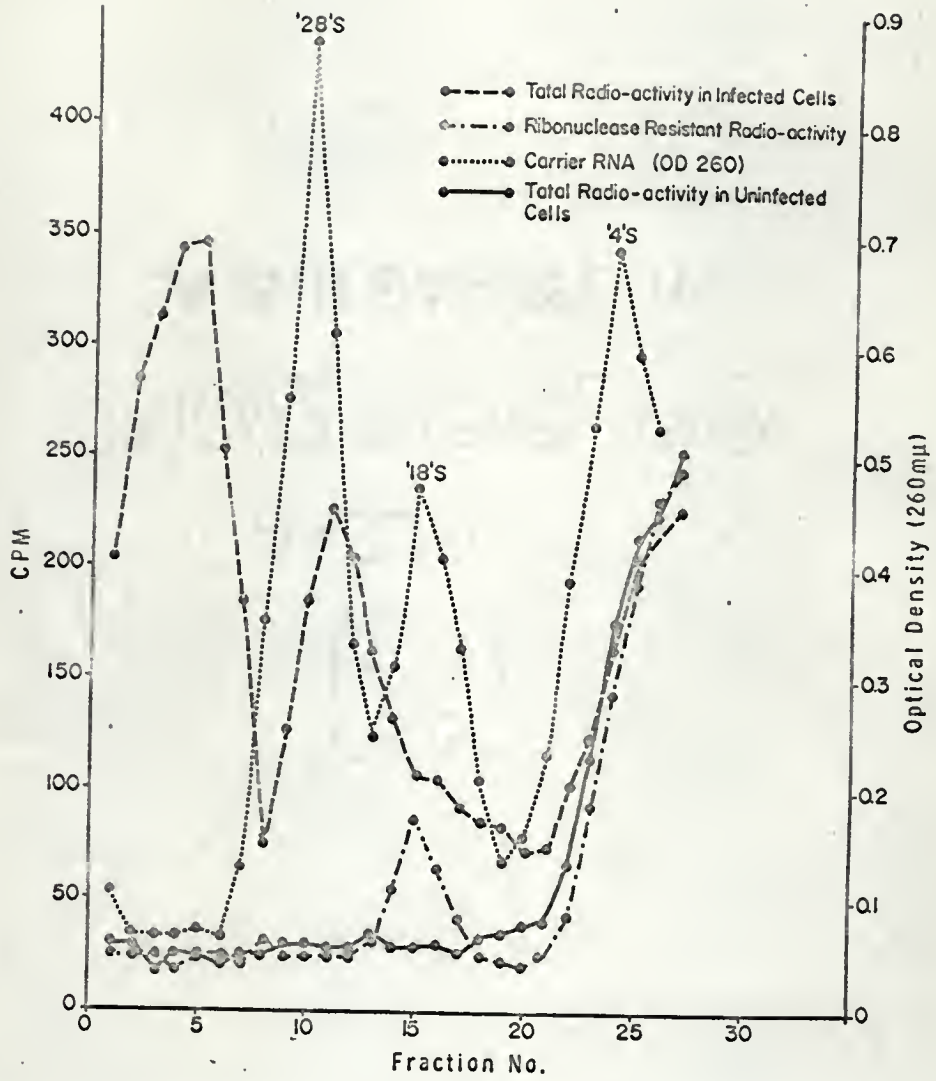

Figure 21. Sucrose gradient analysis of RNA extracted from actinomycin D-treated chick embryo cell cultures infected for 6 hours with Semliki Forest virus. ^{3}H-uridine (20 μC) was added for 45 minutes, and RNA was then extracted. L cell RNA was employed as carrier RNA.

the RNA peak sedimenting at 45 S was infectious and corresponded to the RNA that can be extracted from purified virus particles. The second peak of radioactive RNA sedimenting at 26 S was not infectious but the base composition of this component was similar to that of 45 S RNA and the RNA extracted from virus particles. The 20 S RNA is ribonuclease resistant and thus presumably the double stranded form of the RNA.

The RNA was also extracted at 2, 4 and 6 hours after exposure of the cell cultures to heat inactivated Semliki Forest virus equivalent to 10 pfu/cell. Sucrose gradient analysis of these RNA extracts did not reveal peak of radioactivity at any of the three regions (Figure 22 and 23) indicating that viral specific RNA was not synthesized in the cells exposed to the heat inactivated virus. These cell cultures had a titer of 5.3×10^3 pfu/ml when assayed for infectious virus at 12 hours after infection. The titer of live virus under similar conditions usually varied between 8×10^7 to 2×10^8 pfu/ml.

Pulse-labelling method

Viral specific RNA synthesis in chick embryo cells exposed to live as well as heat inactivated Semliki Forest virus was also studied by the ^{3}H-uridine pulse labelling method. The induced cells, at various periods after the induction, were exposed to 20 μ C of ^{3}H-uridine for 45 minutes and RNA was extracted with perchloric acid as described in Materials and Methods. Chick embryo cell monolayers infected with live virus, at a multiplicity of 10 pfu/cell, showed the synthesis of viral specific RNA which progressively

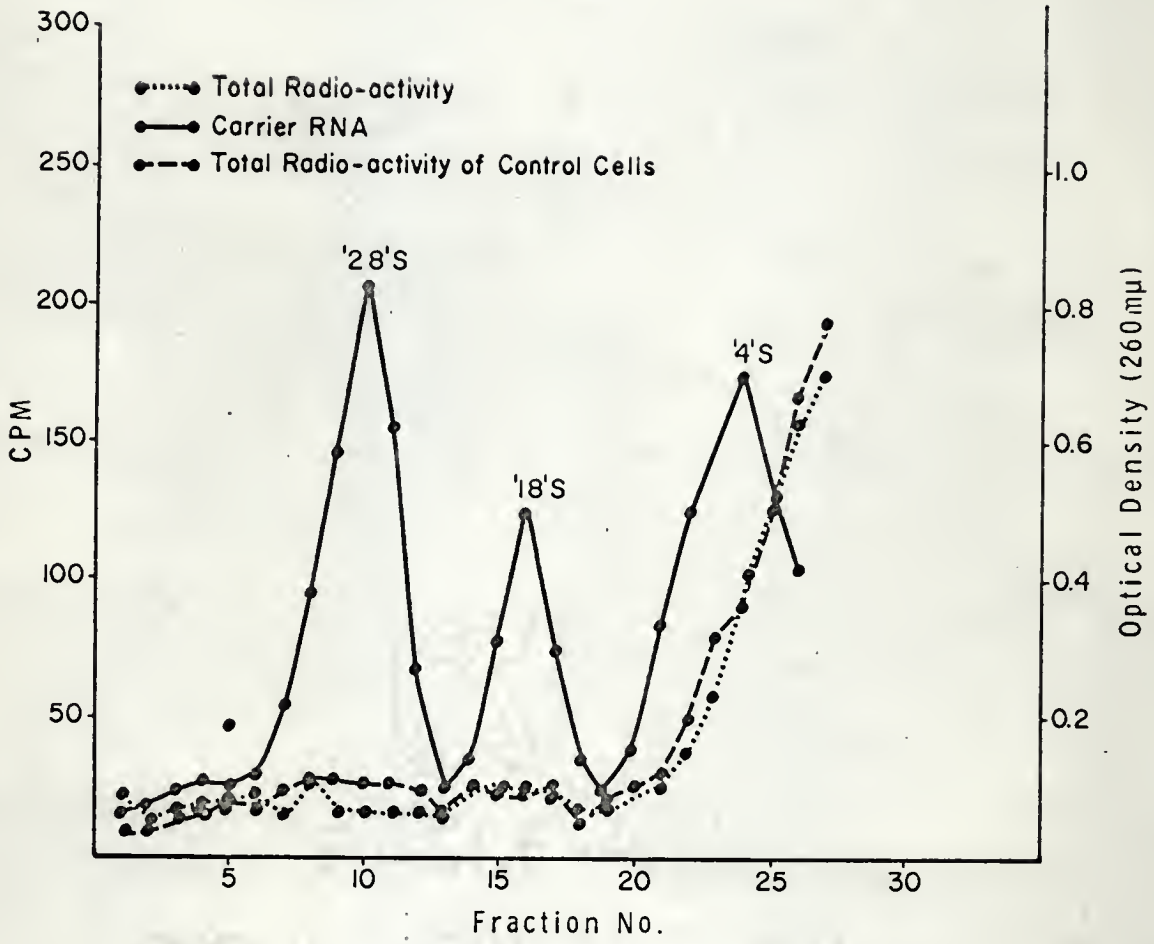

Figure 22. Sucrose gradient analysis of RNA extracted from actinomycin D-treated chick embryo cell cultures exposed for 2 hours with heat inactivated Semliki Forest virus. ^{3}H-uridine (20 μC) was added for 45 minutes and RNA was then extracted. L cell RNA was employed as carrier RNA.

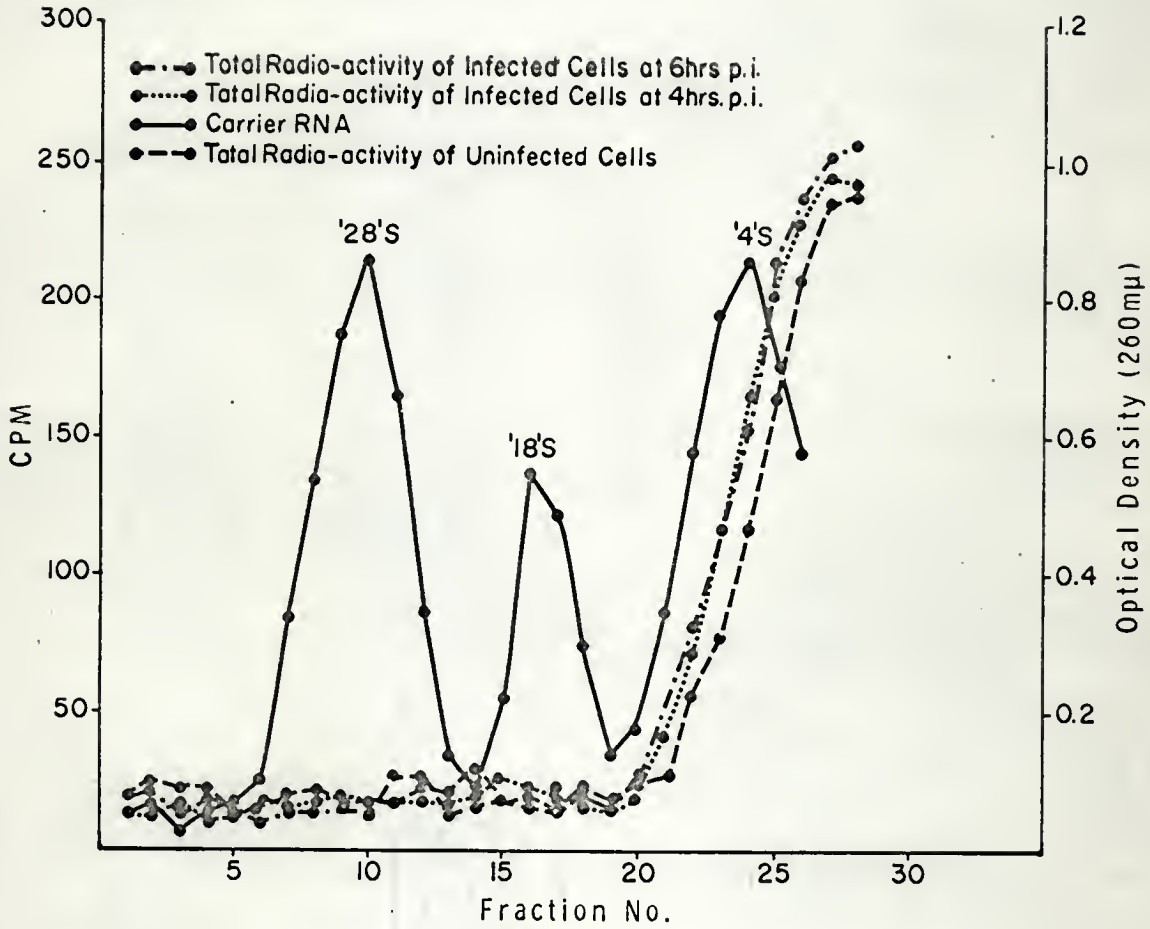

Figure 23. Sucrose gradient analysis of RNA extracted from actinomycin D-treated chick embryo cell cultures exposed to heat inactivated Semliki Forest virus for 4 or 6 hours. ^{3}H-uridine (20 μC) was added for 45 minutes, and RNA was then extracted. L cell RNA was employed as carrier RNA.

increased with respect to time after the infection. Most of the RNA synthesized in the infected cells from 9 hours onward was viral specific (Figure 24). However, no viral specific RNA synthesis was detected when cells were exposed to the heat inactivated virus equivalent to 10 pfu/cell. Virus directed RNA synthesis also could not be detected when cells were exposed to the heat inactivated virus for 7 hours in the presence of 20 μ C of ^{3}H-uridine.

Association of Interferon with Ribosomes

The antiviral activity of interferon seems to depend upon the de novo synthesis of another protein in the exposed cells (Taylor, 1964; Lockart, 1964; Levine, 1966). This new protein, often referred to as translation inhibitory protein (TIP), apparently attaches to the ribosomes and renders these ribosomes incapable of translating the viral messenger RNA (Marcus and Salb, 1966; Carter and Levy, 1967). However, it is not known whether TIP alone, or in conjunction with interferon, produces the change in ribosomal function. The study of ribosomes from cells exposed to interferon alone or to interferon in the presence of actinomycin D cannot answer this question since in the former case the two factors cannot be distinguished from one another and the latter case indicates that interferon alone is not able to mediate the antiviral action in the cells. However, if interferon does play a direct role in the maintenance of virus resistant state in the cells, it is reasonable to assume that it might combine with ribosomes for the expression of antiviral activity.

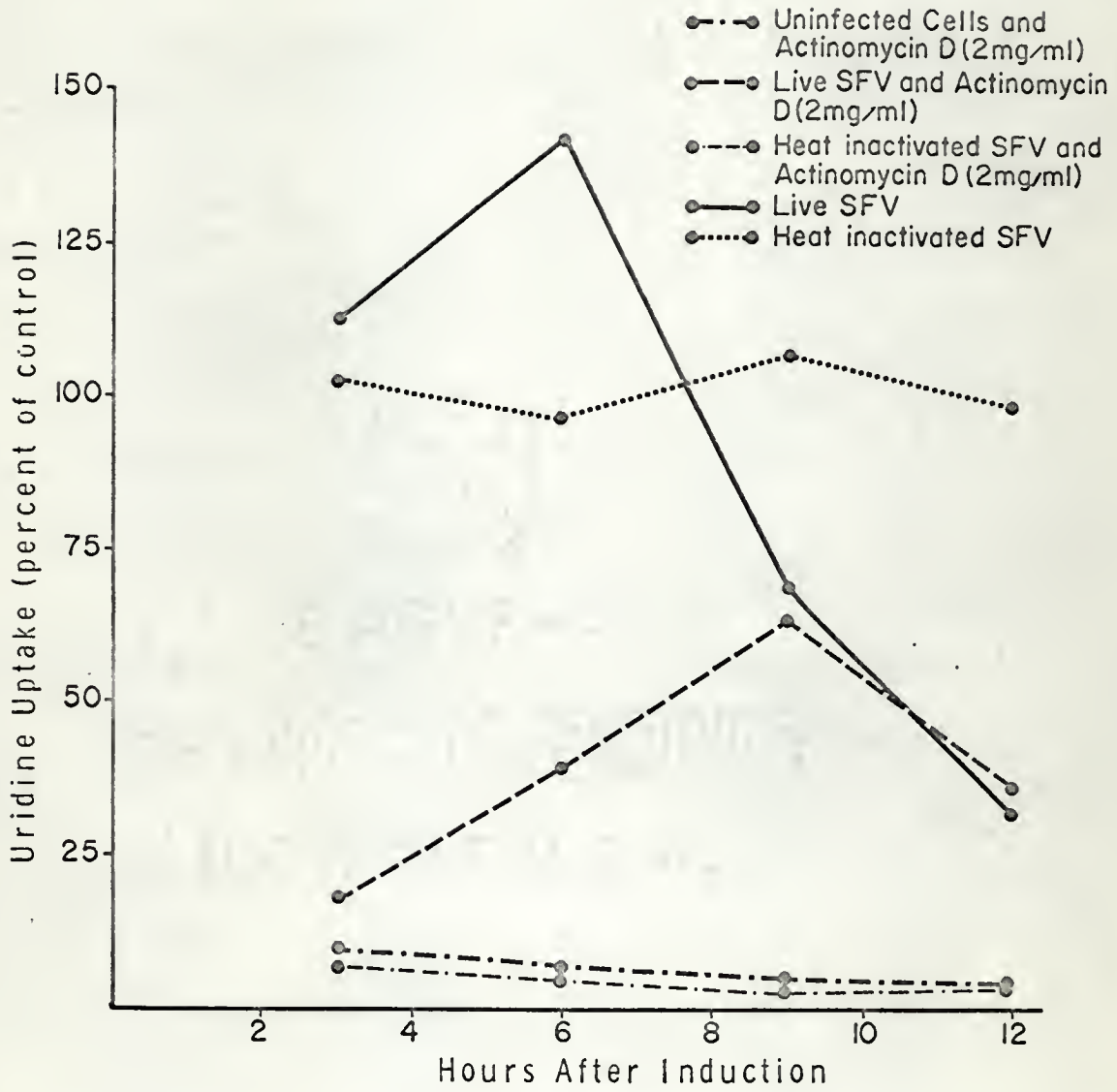

Figure 24. RNA synthesis in chick embryo cell cultures exposed to heat inactivated or live Semliki Forest virus in presence or absence of actinomycin D.

To investigate this possibility, an experiment was performed in which chick embryo and mouse liver ribosomes were examined for their ability to combine with a chick interferon preparation which had been partially purified by centrifugation at 105,000xg for 3 hours. Chick embryo ribosomes (5 OD_{260} units) were mixed with 10 PDD_{50} units of chick interferon and incubated for 30 minutes in a 37°C water bath. After incubation, the ribosomes were removed by centrifugation at 105,000xg for 3 hours and the supernatant fluid was assayed for residual interferon activity. Similar experiments were performed employing chick interferon with mouse liver ribosomes, and also using L cell interferon with mouse liver ribosomes or with chick embryo ribosomes. As presented in Table 4, the results show that almost all of the interferon was recovered after incubation with ribosomes indicating that chick interferon did not bind specifically to chick embryo ribosomes or nonspecifically to mouse liver ribosomes. These studies were extended to include L cell interferon which did not combine with either mouse liver ribosomes or with chick embryo ribosomes.

However, the possibility exists that interferon may bind to one of the ribosomal sub-units but the attachment site is no longer available when '60 S' and '40 S' sub-units combine to form the '80 S' ribosomal unit. To test this possibility, chick embryo ribosomes were separated into '60 S' and '40 S' sub-units as described under Materials and Methods. Two OD_{260} units of chick embryo '40 S' ribosomal

TABLE 4

ASSOCIATION OF CHICK INTERFERON WITH MOUSE AND CHICK EMBRYO RIBOSOMES

Source of Interferon	Source of Ribosomes	PDD_{50} Units of Interferon Added	PDD_{50} Units of Interferon Recovered
Chick Embryo Cells	Chick Embryo (80S)	10	9.7
Chick Embryo Cells	Mouse Liver (80S)	10	10.4
L Cells	Mouse Liver (80S)	10	10.3
L Cells	Chick Embryo (80S)	10	10.1

sub-units were mixed with 10 PDD_{50} interferon units, incubated at 37°C for one hour and ribosomes removed by centrifugation. Similarly, chick interferon was also incubated with '60 S' sub-unit as well as with '80 S' ribosomes. As shown in Table 5, complete recovery of interferon was made after incubation either with '60 S' sub-unit or with '40 S' sub-unit. Thus the results indicate that interferon does not combine with either of the ribosomal sub-units.

TABLE 5

ASSOCIATION OF CHICK INTERFERON WITH RIBOSOMAL SUBUNITS

Source of Interferon	Chick Ribosomes	PDD_{50} Units of Interferon Added	PDD_{50} Units of Interferon Recovered
Chick	60 S	10	9.9
Chick	40 S	10	9.8
Chick	80 S	10	10.1

DISCUSSION

Interferon Production

The dependence of interferon production, in various cell-virus systems, on multiplicity of infection has been well documented in the literature. In many cases, lower multiplicities of infection induce maximum amounts of interferon while higher multiplicities of infection result in reduced yields of interferon (De Mayer and De Somer, 1962; Gifford, 1963; Aurelian and Roizman, 1965; Toy and Gifford, 1967a). In the present study, the highest yield of interferon was obtained when CE cell cultures were infected with SFV at a multiplicity of about 0.1 pfu/cell. Multiplicities of 1 pfu/cell or more of this virus resulted in considerably lower production of interferon. These findings are in agreement with those of Toy and Gifford (1967a).

When two different multiplicities, i.e., 10 pfu/cell and 0.1 pfu/cell were employed, not only were the 24-hour yields different but there were striking differences in the appearance of interferon in CE cell cultures as a function of time after induction. The increase in the yield of interferon between 8 to 24 hours after the infection was roughly linear in cultures infected with a multiplicity of 0.1 pfu/cell. The rate of appearance of interferon in culture medium during this period varied between 0.9 to 1.1 PDD_{50} units/hour/10^6 cells in

different experiments. A similar linear increase in interferon production has been reported by Heller (1963) and Toy and Gifford (1967b) employing chikungunya virus at a multiplicity of 0.1 pfu/cell in CE cell cultures. However, the total amount of interferon produced by SFV during the 24-hour period, as well as its rate of appearance, was lower than that of the chikungunya-CE cell system. When interferon was induced with SFV at 10 pfu/cell, the total yield of interferon varied between 14 to 30% of that induced by 0.1 pfu/cell, and all of the interferon was produced within 10 hours following the infection.

In the current study, when yields were measured 36 hours after infection by SFV, there was some reduction in the amount of interferon as compared to the 24-hour yield. Similar results have been reported by Toy and Gifford (1967b) in the chikungunya-CE cell system. They reported that the disappearance of interferon from the medium was due to its adsorption by the cells since there was no evidence for the presence of proteolytic enzyme(s) in the culture medium to account for the loss. Moreover, the initial rate of disappearance of interferon was comparable to the rate of loss observed when the interferon was added to the uninfected cell cultures. A similar mechanism probably accounted for the decrease in yields of interferon at the 36 hours post-infection period in cells infected with SFV.

The mechanism of inhibition of interferon production by viruses under certain conditions has not been well defined. However, in several cell-virus systems, the production of interferon is dependent

on the continuation of macromolecular synthesis, especially RNA, during the initial stages of viral infection. The necessity of RNA synthesis also becomes apparent by considering the available data which suggest that synthesis of interferon occurs de novo and requires the formation of a new messenger RNA from information encoded into the cellular DNA (reviewed by Burke, 1966).

Aurelian and Roizman (1965) reported that interferon production was partially inhibited in dog kidney cultures infected with 100 pfu/cell of herpes virus (MPdk⁻strain) which caused a rapid decline in cellular RNA synthesis. However, in cell cultures infected with 12 pfu/cell of this virus, the decrease in the cellular RNA synthesis was delayed up to 5 to 6 hours after infection and 70% more interferon was produced. The interpretation of this data is difficult since the RNA synthesis determinations included both viral messenger RNA formation as well as cellular RNA synthesis and it is difficult to correlate interferon production with the severity of the inhibition of cellular RNA synthesis in the infected cells.

Wagner and Huang (1966) observed that interferon synthesis was inhibited in NDV induced Kreb-2 cells when superinfected with virulent VSV at a multiplicity of 50 pfu/cell. Vesicular stomatitis virus infection caused a rapid decline in the rate of cellular RNA synthesis. Interferon synthesis was inhibited when Kreb-2 cells were superinfected within 4 hours after NDV infection and not at 6 or 8 hours. Although VSV caused considerable inhibition of cellular RNA synthesis, it is somewhat difficult to relate this effect to inhibition of

interferon synthesis, not only because of the complexities of the dual infection but also because VSV inhibits cellular DNA and protein synthesis.

In the present study, the differentiation between viral and cellular RNA synthesis was achieved by employing actinomycin D which selectively inhibits cellular RNA synthesis but has no inhibitory effect on SFV replication or viral RNA synthesis (Taylor, 1964). The concentration of actinomycin D (2 μg/ml) employed has been reported to have no effect on cellular DNA synthesis (Kirk, 1960). Under the conditions employed, i.e., when interferon production was minimal (10 pfu/cell of SFV), the total RNA synthesis at 7 hours after infection equalled that of control cells but cellular RNA synthesis was suppressed to 30% of that of uninfected cells (Figure 14). Interferon production ceased at a time when virus synthesis was near completion and cellular RNA synthesis was declining at a rapid rate. These results are in variance with those of Taylor (1965) who reported a nearly linear increase in interferon production for 26 hours in chick embryo cells infected with 10 pfu/cell of Semliki Forest virus. The author reported that the total RNA synthesis of infected cells was similar to controls at 6 to 7 hours, at which time 70 to 90% of the RNA synthesized in the infected cells was of viral origin and indicated a profound inhibition of cellular RNA synthesis. The discrepancy between Taylor's result and those reported here remained unexplained.

In the present studies, interferon synthesis was barely detectable at 5 hours and continued up to 8 to 10 hours post infection

indicating that RNA synthesis was not shut off early enough to completely prevent the interferon specific messenger RNA from being synthesized. Thus, infected cells must be producing both virus and interferon in the initial stages of infection. Termination of interferon synthesis at about 8 hours after infection, even after the formation of interferon specific messenger RNA, apparently contradicts the finding of Wagner (1965) and Wagner and Huang (1966). They found that messenger RNA for interferon, in Newcastle disease virus induced Kreb-2 carcinoma cells, is formed early in the induction period and is relatively stable for nearly 10 hours. However, SFV is a cytopathic virus and cell killing would terminate interferon synthesis even though m-RNA was still present. NDV infection of Kreb-2 carcinoma cells does not lead to cell death and thereby permits the continued functioning of interferon specific messenger RNA. Furthermore, the interferon specific messenger RNA induced by Semliki Forest virus in chick embryo cells may be less stable. This possibility is supported by the observation that interferon synthesis, induced by the heat inactivated SFV, stopped within 12 hours after induction. Similarly, Burke and Walters (1966) reported that interferon synthesis was completed in 10 hours in chick embryo cells induced by Semliki Forest virus under restrictive conditions when virus replication did not occur, but they used elevated temperatures which may have other effects on the stability of interferon specific messenger RNA.

A prolonged synthesis of interferon in cells infected with SFV at a multiplicity of 0.1 pfu/cell was observed and found to be due to

subsequent infection and induction of additional cells by progeny virus. Interferon production stopped at 12 hours after the induction when cells were infected with 0.1 pfu/cell and immune serum against Semliki Forest virus was added before progeny virus appeared. In cell cultures infected under identical conditions but receiving normal rabbit serum, interferon production continued to increase beyond 18 hours after infection. The obvious explanation for such differences in interferon production is that approximately 10% of the cells were initially infected. The cells exposed to live virus produced infectious virus particles and small amounts of interferon. After the first replication cycle, the remaining cells were infected and induced further synthesis of interferon which continued beyond 18 hours. However, in cell cultures containing immune serum, the progeny virus was neutralized and the remaining cells were not infected and thus an earlier termination of interferon synthesis was observed. Additional evidence for this hypothesis is provided by the observation that cell cultures exposed to heat inactivated virus equivalent to 0.1 pfu/cell induced interferon production up to 7 to 8 hours and this yield was comparable to the live virus at that time under identical conditions. However, further synthesis of interferon does not occur because heat inactivated virus is unable to replicate and thus the majority of the cells remain uninfected and uninduced.

An intriguing question is why more interferon synthesized in cell cultures infected at a lower multiplicity than with a high multiplicity. The reason for the increased yield of interferon is not

obvious since the same number of cells were employed in both cases and eventually all the cells would be infected and induced. At this low multiplicity of infection, more than one cycle of virus replication was required for the infection of all the cells which would result in prolonged interferon synthesis but would not explain the increased yield. However, it has been reported by several investigators (Isaacs and Burke, 1958; Friedman, 1966b) that pretreatment of the cells with interferon before the addition of virus enhances the subsequent production of interferon. Thus, at lower multiplicity of infection, interferon produced in the first cycle could enhance the production of interferon in cells infected during the subsequent cycle of viral replication which may result in higher yield of interferon. The other possibility is that inactive virus particles in the virus preparations also induce interferon production, and since such virus particles may not inhibit host macromolecular synthesis, interferon production would be prolonged and enhanced. Therefore, the effect of heat inactivated virus on interferon and host macromolecular synthesis were studied.

Cell monolayers exposed to heat inactivated virus equivalent to 10 pfu/cell produced considerably higher amounts of interferon than the cell cultures infected with an identical amount of live virus. The total yield of interferon was obtained within 12 hours of the exposure of the cell cultures to heat inactivated virus. This higher yield of interferon with heat inactivated virus seems to be due to an enhanced as well as prolonged synthesis of interferon. These effects may be

explained by the observation that heat inactivated virus does not shut off cellular RNA synthesis for the first 15 hours after exposure of the cells. However, even with heat inactivated virus, interferon synthesis is eventually terminated within 12 hours. This may be due to stability characteristics of interferon specific messenger RNA and/or destruction of the inducer in the cell. Interferon may also be responsible for the inhibition of its own synthesis. It has been reported (Vilcek and Rada, 1962; Paucker and Cantell, 1963; Lockart, 1963; Taylor, 1964) that large amounts of interferon in cell culture would inhibit subsequent interferon production when such cells are exposed to an interferon inducer. Thus, such a mechanism could account for the termination of interferon synthesis in cells exposed to heat inactivated virus.

The type of induction initiated by the heat inactivated virus seems identical to that of live Semliki Forest virus since interferon production, in both cases, was almost completely inhibited by pretreatment of cells with actinomycin D. Similar results have been reported by Gifford and Heller (1963) who found that heat-inactivated virus, as well as live chikungunya virus, induced interferon synthesis in chick embryo cells which was inhibited by pretreatment of cells by actinomycin D. These results suggest that heat inactivated as well as live SFV elicit interferon production by inducing de novo protein synthesis in chick embryo cells. The presence of actinomycin D inhibited the formation of interferon specific m-RNA and thus prevented the production of interferon in these cells.

Several workers (Hilleman, 1969; Colby and Chamberlin, 1969) have suggested that RNA viruses induce interferon production by synthesizing double stranded, viral RNA which then acts as the inducing agent. The supportive evidence for this suggestion comes from the observation that double stranded, but not single stranded, RNA from synthetic (Field et al., 1967, 1968; Falcoff and Bercoff, 1968; Vilcek et al., 1968) as well as from natural sources (Field et al., 1968; Hilleman, 1969; Falcoff and Falcoff, 1969) induce interferon production in various cell systems. However, in our experiments, viral specific, double stranded RNA could not be detected in the cells induced by heat inactivated Semliki Forest virus. It is concluded, therefore, that input, single stranded, viral RNA is able to induce interferon production and the formation of double stranded RNA is not essential for the induction event. Dianzani (1969) has reported that in L cells, infected with Newcastle disease virus and simultaneously treated with cyclohexamide or p-flurophenylalanine, interferon was produced after the removal of protein synthesis inhibitor and the addition of actinomycin D. It was concluded that in the presence of protein synthesis inhibitor no viral replication occurred but interferon specific messenger RNA was synthesized; therefore, parental viral RNA or protein(s) or both were the inducers for interferon synthesis. However, these results are apparently contrary to the findings of Skehel and Burke (1968b) who found that Semliki Forest virus induced interferon production in chick embryo cell cultures at 42°C

provided the virus was allowed to first incubate with the cells for a 1 to 2 hour period at 36°C. Under these conditions, progeny virus was not synthesized but during the cell-virus interaction at 36°C for 1 to 2 hours, the infected cells synthesized a mixture of double and single stranded viral RNA and it was suggested that synthesis of the double stranded RNA by the virus may be the initial step required for the induction of interferon synthesis. However, it is possible that mechanisms of interferon induction by viruses and double stranded RNA may not be the same. There are certain differences in the process of interferon production by these two groups of inducing agents which support the concept that double stranded RNA preparations release preformed interferon (Finkelstein, Bausek and Merigan, 1968; Youngner and Hallum, 1968) whereas viral induced interferon production requires de novo protein synthesis (Wagner and Huang, 1965; Burke, 1966; Paucker, 1969). The induction of interferon formation by double stranded RNA from several bacterial and animal viruses (Field et al., 1968; Hilleman, 1969) apparently indicates that this may be the process by which RNA viruses induce interferon production. But the possibility exists that interferon induction by viruses include both de novo synthesis as well as the release of preformed interferon and that production of interferon by various double stranded RNA preparations represent the latter response only.

Several investigators have found that certain single stranded RNA preparations are capable of inducing interferon production (Baron et al., 1968; Finkelstein, Bausek and Merigan, 1968). It has been

reported by De Clercq and Merigan (1969a) that the single stranded homopolymers, polyriboguanylic acid, polyriboinosinic acid and polyriboxanthylic acid at neutral pH and polyriboadenylic acid and polyribocytidylic acid at an acid pH can induce small amounts of interferon. The inducibility characteristic was related to the stability of the secondary structure of homopolymers as indicated by their high temperature of melting (Tm) values. Therefore, the other possible interpretation of our data is that the mechanism of interferon induction by viruses and RNA preparations may be the same but single stranded, viral RNA acts as an efficient inducing agent. The inducibility characteristic of viral RNA may be related to one or more of the physico-chemical properties of viruses. These may include 1) better penetrability of virion and thus its RNA into the cells; 2) probable protection of RNA by viral coat against ribonucleases; 3) a possible stable, secondary structure of viral RNA; and 4) an inherent, higher affinity of viral RNA for a hypothetical receptor site which recognizes the inducer and triggers on the mechanism for interferon production inside the cell.

Lockart et al. (1968) employed temperature sensitive mutants of Sindbis virus to investigate viral event(s) necessary for the induction of interferon synthesis in chick embryo cells. Three RNA^+ strains (able to synthesize viral RNA but not infectious virus particles at 42°C) produced interferon at 29°C but not at 42°C. Since the defect in these temperature sensitive mutants apparently result from the synthesis of viral proteins which are not functional at restrictive

temperatures, it was suggested that viral protein(s), or the process for which they are necessary, is required for interferon production. The possible role of viral protein(s) synthesis in the process of interferon induction by heat inactivated Semliki Forest virus, cannot be excluded in our experiments. This is because the exact mechanism of heat inactivation of viruses is not well understood. The kinetic data obtained by Ginoza (1958) suggested that the spontaneous decay of tobacco mosaic virus--ribonuclec acid (TMV-RNA) is related to the inherent susceptibility of its phosphodiester bonds to heat induced hydrolysis. However, the phosphodiester bonds of single and double stranded DNA are stable under thermal treatment at neutral pH but in either form is relatively sensitive to depurination (Ginoza et al., 1964). The TMV-RNA was found to be 30 times more sensitive to heat inactivation at 37°C than single stranded DNA of comparable size. On the basis of such observations, it has been suggested (Ginoza, 1968) that the inactivation of viruses at lower temperature is due to some simple covalent bond scission occurring in the nucleic acid. It is possible, however, that part of the multicistronic genome of the heat inactivated virus may remain functional and act as its own messenger RNA so that viral protein(s) synthesis in chick embryo cells exposed to heat-inactivated Semliki Forest virus can not be ruled out.

The results show that, under the experimental conditions employed, interferon does not combine specifically with homologous

ribosomes or nonspecifically with heterologous ribosomes. The failure to detect association could have been due to the presence of a greater number of interferon molecules than ribosomes, especially if only one interferon molecule could react with one ribosome. In such a case, only a fraction of interferon molecules would attach to the ribosomes and this small loss would not be detectable in the interferon assay. However, the number of ribosomes (4.4×10^{13} molecules/5 OD_{258} units) far exceeded the number of interferon molecules (9.0×10^{10}/10 PDD_{50} units) in the experiments and thus excluded such a possibility. The calculations for the number of ribosome molecules were based on the extinction coefficient ($E^{1\%}_{20}$ 135 ± 3/cm at 260 mμ) and molecular weight ($5.0 \pm 0.2 \times 10^{6}$) of guinea pig liver ribosomes as described by Tashiro and Siekevitz (1965). These values should be applicable for mouse and chick embryo ribosomes since the S values of the ribosomes from all three animal species are comparable. The calculations for interferon molecules were based on the observation that each PDD_{50} unit protects nearly 2×10^{6} cells and according to Merigan, Winget and Dixon (1965), in most purified preparations a maximum of 4,500 molecules of interferon would be required to protect one cell.

These results suggest that interferon does not directly affect the ribosomal function which is necessary for the viral resistant state in the cell. Similarly, Heller (1968) observed that the presence of interferon did not affect the rate of amino acid incorporation into a cell-free protein synthesis system. It seems that the role of

interferon is to induce the synthesis of TIP in the exposed cells which then combines alone with ribosomes so that these ribosomes are capable of forming polysomes with cellular messenger RNA but bind poorly to viral messenger RNA, thus inhibiting viral RNA directed protein synthesis. However, it is possible that interferon does possess the ability to combine with ribosomes and the failure to detect such an association in the present experiments may be due to several factors. These include 1) the temperature (37°C) and pH (7.2) of the mixture may not be suitable for the association of interferon with ribosomes; 2) the interferon preparation might have some factor(s) which inhibited the attachment of interferon to the ribosomes; 3) it has been reported (Brown and Doty, 1968) that washing of ribosomes with 1 M ammonium chloride solution results in the removal of 3 proteins and one of these proteins is required for the attachment of f2 RNA to the ribosomes. It is possible, therefore, that during the isolation and purification of the ribosomes in the present experiments, some protein(s) might have been removed and the prior presence of these protein(s) may be essential for the attachment of interferon to the ribosomes; and 4) finally, interferon may attach only when TIP is already bound to the ribosomes or that TIP interferon must interact before the complex can bind with the ribosome. The direct role of interferon in the maintenance of viral resistant state can be definitely established only under the conditions in which purified TIP and interferon could be added to the ribosomes, and to analyse the

affect of each factor alone and both combined in the interaction and translation of viral and cellular messenger RNA *in vitro*.

SUMMARY

Interferon production in chick embryo cell cultures infected with different multiplicities of Semliki Forest virus was studied. Input multiplicities of 1 pfu/cell or more resulted in low yields of interferon while considerably higher yields were obtained when cells were infected with an input multiplicity of 0.1 pfu/cell. At an input multiplicity of 10 pfu/cell, interferon production stopped at 10 hours after the infection whereas interferon synthesis continued beyond 18 hours following infection with 0.1 pfu/cell. Prolonged interferon synthesis with the lower multiplicity was shown to be due to continuous induction by progeny virus. When cells were exposed to sufficient virus to infect each cell, much less interferon was found with live virus than with inactivated virus. It was concluded that early termination of interferon synthesis with higher multiplicities of infection was due to the rapid and profound inhibition of cellular RNA synthesis of the infected cells. This conclusion was based on the evidence that Semliki Forest virus at a multiplicity of 10 pfu/cell caused rapid inhibition of cellular RNA synthesis so that after 8 hours practically all the RNA synthesized in the infected cell was of viral origin. Chick embryo cell cultures exposed to 10 pfu/cell equivalent amount of heat inactivated Semliki Forest virus produced considerably higher amounts of interferon with no concomitant inhibition of

the cellular RNA synthesis up to 12 hours following the induction.

It seems that the mechanism of appearance of interferon in chick embryo cells exposed to heat inactivated as well as live Semliki Forest virus is due to de novo synthesis of protein in the induced cells since the presence of actinomycin D in cell cultures completely inhibited the appearance of interferon in both cases. No double stranded viral specific RNA synthesis could be detected in chick embryo cells exposed to 10 pfu/cell equivalent amount of heat inactivated Semliki Forest virus. Therefore, it was concluded that single stranded input viral RNA in itself is capable of inducing interferon production in the induced cells. However, the role of viral protein(s), if any, in interferon induction could not be ascertained.

Under the experimental conditions employed, we could not detect the association of interferon with ribosomes of homologous or heterologous species. Binding of interferon could also not be detected with 40 S and 60 S ribosomal units of the homologous species. Therefore, it seems likely that interferon does not play a direct role in the maintenance of a viral resistant state in the cells.

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